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April 4, 2008

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Document Control Office (7407M)
U.S. Environmental Protection Agency
Attn: TSCA Section 8(e) Coordinator
Office of Pollution Prevention and Toxics
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001

Dear Section 8(e) Coordinator:

Re: Carbamic acid, [(butylthio)thioxomethyl]-, butyl ester, **File No. 8EHQ-08-17077.**

Enclosed is the final report for an Acute Toxicity Study in freshwater algae Desmodesmus subspicatus, compliant with OECD 201 Guidelines, on carbamic acid, [(butylthio)thioxomethyl]-, butyl ester, CAS 1001320-38-2. Concentrations tested were 0.015, 0.048, 0.15, 0.48 and 1.5 mg/l for 72 h at 24°C. The material is minimally soluble in the aqueous test media, and was found to be unstable over the time period of the study. Quantitative analysis was performed on the dose concentrations utilized in the study, with the finding that the concentrations decreased up to 70% during the study. Accounting for this decrease, the ErC50 value (0 -72 h) was calculated to be 0.15 mg/l, (95% confidence limits 0.10 - 0.23 mg/l); the EyC50 (0 -72 h) was 0.10 mg/l, (95% confidence limits 0.007 – 0.014 mg/l); and the EbC50 (0 – 72 h) was 0.014 mg/l (95% confidence limits 0.010 – 0.021 mg/l). The NOEC was 0.018 mg/l.

As this correspondence contains confidential business information, a sanitized version is attached.

If you have any additional questions or comments, please contact me at .

Sincerely,

SafePharm Laboratories

ALGAL GROWTH INHIBITION TEST

SPL PROJECT NUMBER: 2337/0007

AUTHORS: H Vryenhoef
D M Mullee

STUDY SPONSOR:

TEST FACILITY:

Safepharm Laboratories Limited
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD
UK

Telephone: +44 (0) 1332 792896

Facsimile: +44 (0) 1332 799018

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress. In addition, inspection of general facilities not specifically related to this study are done monthly or annually in accordance with QA Standard Procedure.

This report has been audited by Safepharm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	18 July 2007	Standard Test Method Compliance Audit
	04 December 2007	Test Material Preparation
	10 December 2007	Test System Preparation
	17 December 2007	Exposure
	07 December 2007	Assessment of Response
	03, 06 December 2007	Chemical Analysis
§	21 February 2008	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 27 FEB 2008

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**

Head of Department:	JR Pateman CBiol MIBiol DipRQA ACQI FRQA
Deputy Head of Department:	JM Crowther MIScT MRQA
Senior Audit Staff:	JV Johnson BSc MRQA; G Wren ONC MRQA

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GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.

H. Vryenhoef ..... Date: 27 FEB 2008

H Vryenhoef BSc
Study Director

The analytical data presented in this report were compiled by me or under my supervision and accurately reflect the data obtained.

..... Date: 27 FEB 2008

D M Mullee CChem MRSC
Director of Analytical Services

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ALGAL GROWTH INHIBITION TEST

SUMMARY

Introduction. A study was performed to assess the effect of the test material on the growth of the green alga *Desmodesmus subspicatus*. The method followed that described in the OECD Guidelines for Testing of Chemicals (2006) No 201, "Freshwater Alga and Cyanobacteria, Growth Inhibition Test" referenced as Method C.3 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Methods. A determination of the General Physico-Chemical Properties study conducted on the test material (Safepharm Laboratories Project Number: 2337/0003) showed the water solubility value of the test material was 2.58 mg/l. A pre-study media preparation trial indicated that a dissolved test material concentration of approximately 1.5 mg/l was obtained from a saturated solution method of preparation indicating this to be the limit of water solubility of this material under test conditions.

Following a preliminary range-finding test *Desmodesmus subspicatus* was exposed to solutions of the test material at nominal concentrations of 0.015, 0.048, 0.15, 0.48 and 1.5 mg/l (three replicate flasks per concentration) for 72 hours, under constant illumination and shaking at a temperature of 24 ± 1°C. The test material solutions were prepared by stirring an excess (50 mg/l) of test material in culture medium using a propeller stirrer at approximately 1500 rpm at a temperature of 21°C for 24 hours. After the stirring period any undissolved test material was removed by filtration (0.2 µm Sartorius Sartopore filter, first approximate 1 litre discarded in order to pre-condition the filter) to produce a saturated solution of the test material with a nominal concentration of 1.5 mg/l*. This saturated solution was then further diluted as necessary, to provide the remaining test groups.

Samples of the algal populations were removed daily and cell concentrations determined for each control and treatment group, using a Coulter® Multisizer Particle Counter.

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* Concentration determined by analysis of a saturated solution prepared in an identical manner during the pre-study media preparation trial.

A positive control conducted approximately every six months used potassium dichromate as the reference material. *Desmodesmus subspicatus* was exposed to an aqueous solution of the reference material at concentrations of 0.0625, 0.125, 0.25, 0.50 and 1.0 mg/l (three replicate flasks per concentration) for 72 hours, under constant illumination and shaking at a temperature of 24 ± 1°C.

Samples of the algal populations were removed daily and cell concentrations determined for each control and treatment group, using a Coulter® Multisizer Particle Counter.

Results. In terms of growth rate, exposure of *Desmodesmus subspicatus* to the test material gave an E_rC_{50} (0 - 72 h) value of 0.31 mg/l; 95% confidence limits 0.27 – 0.36 mg/l. The Lowest Observed Effect Concentration based on inhibition of growth rate was 0.048 mg/l and the No Observed Effect Concentration was 0.015 mg/l.

In terms of yield, exposure of *Desmodesmus subspicatus* to the test material gave an E_yC_{50} (0 - 72 h) value of 0.11 mg/l; 95% confidence limits 0.084 – 0.15 mg/l. The Lowest Observed Effect Concentration based on yield was 0.048 mg/l and the No Observed Effect Concentration was 0.015 mg/l.

In terms of biomass integral (area under growth curve), exposure of *Desmodesmus subspicatus* to the test material gave an E_bC_{50} (0 - 72 h) value of 0.13 mg/l; 95% confidence limits 0.10 – 0.18 mg/l. The Lowest Observed Effect Concentration based on inhibition of biomass integral was 0.048 mg/l and the No Observed Effect Concentration was 0.015 mg/l.

Analysis of the test preparations at 0 hours showed measured test concentrations to range from 84% to 121% of nominal. Analysis of the test preparations at 72 hours showed a decline in measured test concentrations in the range of less than 1% of nominal to 71% of nominal with the lowest test concentrations exhibiting the greatest decline. This decline was inline with the stability analyses conducted which indicated that the test material was unstable in culture medium over the test duration particularly at the lower test concentrations employed. A further decline in excess of that seen in the stability analyses was considered to be due to possible adsorption of the test material to the algal cells present. Whilst no immediate adsorption was observed in the recovery analyses conducted in the presence of algal cells this does not preclude long-term

adsorption over the test period. Adsorption was not a factor in the stability analyses as no algal cells were present.

Given this decline in measured test concentrations it was considered justifiable to base the results on the geometric mean measured test concentrations in order to give a "worst case" analysis of the data. The E_rC_{50} (0 - 72 h) based on the geometric mean measured test concentrations was 0.15 mg/l; 95% confidence limits 0.10 – 0.23 mg/l, the E_yC_{50} (0 - 72 h) was 0.010 mg/l; 95% confidence limits 0.0070 – 0.014 mg/l, and the E_bC_{50} (0 - 72 h) was 0.014 mg/l; 95% confidence limits 0.010 – 0.021 mg/l. The Lowest Observed Effect Concentration based on inhibition of growth rate, yield and biomass integral was 0.0045 mg/l and the No Observed Effect Concentration was 0.0018 mg/l.

Exposure of *Desmodesmus subspicatus* to the reference material, potassium dichromate, gave an E_rC_{50} (0 - 72 h) of 0.49 mg/l; 95% confidence limits 0.43 – 0.55 mg/l, an E_yC_{50} (0 - 72 h) of 0.22 mg/l; 95% confidence limits 0.19 - 0.24 mg/l, and an E_bC_{50} (0 - 72 h) of 0.23 mg/l; 95% confidence limits 0.21 - 0.27 mg/l. The Lowest Observed Effect Concentration based on inhibition of growth rate, yield and biomass integral were 0.25, 0.125 and 0.125 mg/l respectively and the No Observed Effect Concentrations were 0.125, 0.0625 and 0.0625 mg/l respectively.

ALGAL GROWTH INHIBITION TEST

1. INTRODUCTION

This report contains a description of the methods used and results obtained during a study to investigate the effect of the test material on the growth of the green alga *Desmodesmus subspicatus*. The method followed the recommendations of the OECD Guidelines for Testing of Chemicals (2006) No 201, "Freshwater Alga and Cyanobacteria, Growth Inhibition Test" referenced as Method C.3 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Desmodesmus subspicatus (formerly known as *Scenedesmus subspicatus*) is a freshwater unicellular alga, representative of primary producers found in natural waters and can therefore be considered as an important non-target organism in freshwater ecosystems.

The study was conducted between 29 November 2007 and 14 December 2007.

The positive control (Safepharm Laboratories Project Number: 0039/0941) was conducted between 12 June 2007 and 15 June 2007.

In view of the difficulties associated with the evaluation of aquatic toxicity of poorly water soluble test materials, a modification of the standard method for the preparation of aqueous media was performed. An approach endorsed by several important regulatory authorities in the EU and elsewhere (ECETOC 1996 and OECD 2000), is to expose organisms to a saturated solution of the test material in cases where the test material is of high purity and is poorly soluble in water and in the permitted auxiliary solvents and surfactants. Using this approach, a saturated solution was prepared by stirring an excess (50 mg/l) of test material in culture medium for a period of 24 hours prior to removing any undissolved test material present by filtration (0.2 µm Sartorius Sartopore, first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution of the test material.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	
Description	:	yellow coloured solid
Batch number	:	MR1169
Date received	:	27 September 2007
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor. A Certificate of Analysis for the test material supplied by the Sponsor is given in Appendix 1.

3. METHODS

3.1 Test Species

The test was carried out using *Desmodesmus subspicatus* strain CCAP 276/20. Liquid cultures of *Desmodesmus subspicatus* were obtained from the Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Laboratory, Oban, Argyll, Scotland. Master cultures were maintained in the laboratory by the periodic replenishment of culture medium (Section 3.2). The master cultures were maintained in the laboratory under constant aeration and constant illumination at 21 ± 1°C.

Prior to the start of the test sufficient master culture was added to approximately 100 ml volumes of culture media contained in conical flasks to give an initial cell density of approximately 10^3 cells/ml. The flasks were plugged with polyurethane foam stoppers and kept under constant agitation by orbital shaker (100 – 150 rpm) and constant illumination at 24 ± 1°C until the algal cell density was approximately 10^4 - 10^5 cells/ml.

3.2 Culture Medium

The culture medium used for both the range-finding and definitive tests was the same as that used to maintain the stock culture.

The culture medium is defined in Appendix 2.

3.3 Procedure

3.3.1 Pre-study media preparation trial

A study to determine the General Physico-Chemical properties of the test material (Safepharm Laboratories Project Number: 2337/0003) determined the water solubility of the test material to be 2.58 mg/l. Preliminary solubility work conducted indicated that the test material was practically insoluble in water using traditional methods of preparation e.g. ultrasonication and high shear mixing. A test concentration of 2.0 mg/l (by visual inspection) was obtained using a preliminary solution in dimethylformamide.

Based on this information the test material was categorised as being a 'difficult substance' as defined by the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (OECD 2000). Therefore a media preparation trial was conducted in order to determine the solubility of the test material under test conditions.

3.3.1.1 Saturated solution preparation

An amount of test material (550 mg) was dispersed, in duplicate, in 11 litres of culture medium with the aid of propeller stirring at approximately 1500 rpm at a temperature of 21°C for periods of 24 or 48 hours. After the stirring periods samples were taken for chemical analysis after the following pre-treatments:

- Centrifugation at 10000 *g* for 30 minutes
- Centrifugation at 40000 *g* for 30 minutes
- Filtration through a 0.2 µm Sartorius Sartopore filter (approximately 1 litre discarded in order to pre-condition the filter)
- Filtration through a 0.2 µm Sartorius Sartopore filter (approximately 2 litres discarded in order to pre-condition the filter)

3.3.1.2 Solvent spike preparation

An amount of test material (200 mg) was dissolved in dimethylformamide and the volume adjusted to 10 ml to give a 200 mg/10 ml solvent stock solution. An aliquot (1000 µl) of this stock solution was dispersed in 10 litres of culture medium with the aid of magnetic stirring for

- Untreated
- Centrifugation at 10000 *g* for 30 minutes
- Centrifugation at 40000 *g* for 30 minutes
- Filtration through a 0.2 µm Gelman Acrocap filter (approximately 100 ml discarded in order to pre-condition the filter)
- Filtration through a 0.2 µm Gelman Acrocap filter (approximately 500 ml discarded in order to pre-condition the filter)

The remainder of the 2.0 mg/l test concentration was returned to the magnetic stirrer and stirred for a further 48 hours with samples being taken for analysis after both 24 and 48 hours stirring.

3.3.2 Range-finding test

The test concentrations to be used in the definitive test were determined by a preliminary range-finding test. The range-finding test was conducted by exposing *Desmodesmus subspicatus* cells to a series of nominal test concentrations of 0.015, 0.15 and 1.5 mg/l for a period of 72 hours.

An amount of test material (550 mg) was dispersed in 11 litres of culture medium with the aid of propeller stirring at approximately 1500 rpm at a temperature of 21°C for 24 hours. After 24 hours the stirring was stopped and any undissolved test material was removed by filtration through a 0.2 µm Sartorius Sartopore filter (first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution with a nominal concentration of 1.5 mg/l*. A series of dilutions was made from this saturated solution to give further stock solutions of 0.15 and 0.015 mg/l. An aliquot (250 ml) of each of the stock solutions was separately inoculated with algal suspension (2.4 ml) to give the required test concentrations of 0.015, 0.15 and 1.5 mg/l.

The test was conducted in 250 mg/l glass conical flasks each containing 100 ml of test preparation and plugged with polyurethane foam bungs to reduce evaporation. Two replicate flasks each containing 100 ml of test preparation were used for each control and test concentration.

The control group was maintained under identical conditions but not exposed to the test material.

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* Concentration determined by analysis of a saturated solution prepared in an identical manner during the pre-study media preparation trial.

At the start of the range-finding test a sample of each test and control culture was removed and the cell density determined using a Coulter® Multisizer Particle Counter. The flasks were then plugged with polyurethane foam bungs and incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) provided by warm white lighting (380 – 730 nm) and constantly shaken at approximately 150 rpm for 72 hours.

After 72 hours the cell density of each flask was determined using a Coulter® Multisizer Particle Counter.

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3.3.3 Definitive test

Based on the results of the range-finding test, test material solutions for the definitive test were prepared by stirring an excess (50 mg/l) of test material in culture medium for a period of time and then removing any undissolved test material by filtration. The saturated solution was then further diluted, as necessary, to produce the remaining test groups.

3.3.3.1 Experimental Preparation

Due to the low aqueous solubility and high purity of the test material the test concentrations used in the definitive test were prepared by diluting (with culture medium) a saturated solution prepared from an initial test material dispersion at a concentration of 50 mg/l.

An amount of test material (550 mg) was dispersed in 11 litres of culture medium with the aid of propeller stirring at approximately 1500 rpm at a temperature of 21°C for 24 hours. After 24 hours the stirring was stopped and any undissolved test material was removed by filtration through a 0.2 µm filter (first approximate 1 litre discarded in order to pre-condition the filter) to give a stock solution with a nominal concentration of 1.5 mg/l*.

A series of dilutions was made from this stock solution to give further stock solutions of 0.48, 0.15, 0.048 and 0.015 mg/l. An aliquot (1 litre) of each of the stock solutions was separately inoculated with 5.6 ml algal suspension to give the required test concentrations of 0.015, 0.048, 0.15, 0.48 and 1.5 mg/l.

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* Concentration determined by analysis of a saturated solution prepared in an identical manner during the pre-study media preparation trial.

The concentration and stability of the test material in the test solutions were verified by chemical analysis at 0 and 72 hours (see Appendix 3).

3.3.3.2 Exposure conditions

As in the range-finding test 250 ml glass conical flasks were used. Six flasks each containing 100 ml of solution were used for the control and three flasks each containing 100 ml were used for each treatment group.

The control group was maintained under identical conditions but not exposed to the test material.

Pre-culture conditions gave an algal suspension in log phase growth characterised by a cell density of 7.19×10^5 cells per ml. Inoculation of 1 litre of test medium with 5.6 ml of this algal suspension gave an initial nominal cell density of 4×10^3 cells per ml and had no significant dilution effect on the final test concentration.

The flasks were plugged with polyurethane foam bungs and incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) provided by warm white lighting (380 – 730 nm) and constantly shaken at approximately 150 rpm for 72 hours.

Samples were taken at 0, 24, 48 and 72 hours and the cell densities determined using a Coulter® Multisizer Particle Counter.

3.3.3.3 Physico-chemical measurements

The pH of each control and test flask was determined at initiation of the test and after 72 hours exposure. The pH was measured using a WTW pH 320 pH meter. The temperature within the incubator was recorded daily.

3.3.3.4 Verification of test concentrations

Samples were taken from the control (replicates R_1 - R_6 pooled) and each test group (replicates R_1 - R_3 pooled) at 0 and 72 hours for quantitative analysis. Duplicate samples were taken at 0 hours and stored at approximately -20°C for further analysis if necessary. Sample volumes required for chemical analysis precluded the storage of duplicate samples at 72 hours.

The method of analysis, stability, recovery and test solution analyses are described in Appendix 3.

3.3.4 Evaluation of data

3.3.4.1 Comparison of growth rates

The average specific growth rate for a specified period is calculated as the logarithmic increase in biomass from the equation:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

where:

μ = average specific growth rate from time t_1 to t_n
N_1 = cell concentration at t_1
N_n = cell concentration at t_n
t_1 = time of first measurement
t_n = time of n^{th} measurement

The average specific growth rate over the test duration was calculated for each replicate control and test material vessel using the nominally inoculated cell concentration as the starting value rather than the measured starting value in order to increase the precision of the calculation.

In addition the section by section specific growth rate (days 0-1, 1-2 and 2-3) was calculated for the control cultures and the results examined in order to determine whether the growth rate remained constant.

Percentage inhibition of growth rate for each replicate test material vessel was calculated using the following equation:

$$I_r = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

where:

I_r = percentage inhibition of average specific growth rate

μ_c = mean average specific growth rate for the control cultures

μ_t = average specific growth rate for the test culture

3.3.4.2 Comparison of Yield

Yield is calculated as the increase in biomass over the exposure period using the following equation:

$$Y = N_n - N_0$$

where:

Y = yield

N_0 = cell concentration at the start of the test

N_n = cell concentration at the end of the test

For each test concentration and control the mean value for yield along with the standard deviation was calculated. Percentage inhibition of yield was calculated using the following equation:

$$I_y = \frac{(Y_c - Y_t)}{Y_c} \times 100$$

where:

I_y = percentage inhibition of yield

Y_c = mean value for yield in the control group

Y_t = mean value for yield for the treatment group

3.3.4.3 Comparison of biomass integral

The biomass integral (area under the growth curve) was calculated using the following equation:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

where:

A = area

N_0 = nominal cell concentration at start of test

N_1 = measured cell concentration at t_1
N_n = measured cell concentration at t_n
t_1 = time of first measurement after beginning of test
t_n = time of n^{th} measurement after beginning of test

Percentage inhibition of the biomass integral for each replicate test material vessel was calculated using the following equation:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

where:

I_A = percentage inhibition of the biomass integral
A_c = mean biomass integral for the control cultures
A_t = biomass integral for the test culture

3.3.4.4 Determination of EC_x values

For each individual test vessel (mean values for yield), percentage inhibition (arithmetic axis) was plotted against test concentration (logarithmic axis) and a line fitted by computerised interpolation using the Xlfit software package (IDBS). EC_x values were then determined from the equation for the fitted line.

Where appropriate 95% confidence limits for the EC_{50} values were calculated, using the simplified method of evaluating dose-effect experiments of Litchfield and Wilcoxon (1949).

3.3.4.5 Statistical analysis

One way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955) was carried out on the growth rate, yield and biomass integral data after 72 hours for the control and all test concentrations to determine any statistically significant differences between the test and control groups. All statistical analyses were performed using the SAS computer software package (SAS 1999 - 2001).

3.3.4.6 Geometric mean measured test concentrations

The geometric mean measured test concentrations of the samples were calculated as follows using the measured test concentrations of replicates R_1 - R_3 pooled:

$$GM = \sqrt{C_0 \times C_1}$$

where

GM	=	geometric mean measured test concentration (mg/l)
C_0	=	measured concentration at the start of the test (mg/l)
C_1	=	measured concentration at the end of the test (mg/l)

3.3.5 Positive Control

A positive control (Safepharm Laboratories Project Number 0039/0941) used potassium dichromate as the reference material. An amount of reference material (100 mg) was dissolved in culture medium and the volume adjusted to 1 litre to give a 100 mg/l stock solution from which a series of dilutions were made to give further stock solutions of 10, 2.0, 1.0, 0.50, 0.25 and 0.125 mg/l. An aliquot (250 ml) of each of the 0.125, 0.25, 0.50, 1.0 and 2.0 mg/l stock solutions was separately mixed with algal suspension (250 ml) to give the required test concentrations of 0.0625, 0.125, 0.25, 0.50 and 1.0 mg/l.

The test was conducted in 250 ml glass conical flasks each containing 100 ml of test preparation and plugged with polyurethane foam bungs to reduce evaporation. Six replicate flasks were prepared for the control and three replicate flasks prepared for each test concentration.

The flasks were incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) provided by warm white lighting (380 – 730 nm) and constantly shaken at approximately 150 rpm for 72 hours.

Samples were taken at 0, 28, 52 and 72 hours and the cell densities determined using a Coulter® Multisizer Particle Counter.

3.3.6 Evaluation of data for the positive control

3.3.6.1 Comparison of growth rates

Average specific growth rates and inhibition of growth rate were calculated as in Section 3.3.4.1.

3.3.6.2 Comparison of Yield

Yield and percentage inhibition of yield were calculated as in Section 3.3.4.2.

3.3.6.3 Comparison of biomass integral

The biomass integral (area under the growth curve) and inhibition of the biomass integral were calculated as in Section 3.3.4.3.

3.3.6.4 Determination of EC_x values

For each individual test vessel (mean values for yield), percentage inhibition (arithmetic axis) was plotted against test concentration (logarithmic axis) and a line fitted by computerised interpolation using the Xlfit software package (IDBS). EC_x values were then determined from the equation for the fitted line.

Where appropriate 95% confidence limits for the EC_{50} values were calculated, using the simplified method of evaluating dose-effect experiments of Litchfield and Wilcoxon (1949).

3.3.6.5 Statistical analysis

One way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955) was carried out on the growth rate, yield and biomass integral data after 72 hours for the control and all test concentrations to determine any statistically significant differences between the test and control groups. All statistical analyses were performed using the SAS computer software package (SAS 1999 - 2001).

3.4 Validation Criteria

The results of the test are considered valid if the following performance criteria are met:

- The cell concentration of the control cultures must increase by a factor of at least 16 over the test period.

- The mean of the coefficients of variation of the section by section daily growth rates in the control cultures during the course of the test (days 0-1, 1-2 and 2-3, for 72-Hour tests) must not exceed 35%.
- The coefficient of variation of the average specific growth rate in replicate control cultures must not exceed 7%.

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Pre-Study Media Preparation Trial

A determination of the General Physico-chemical properties study conducted on the test material (Safepharm Laboratories Project Number: 2337/0003) showed the water solubility value of the test material was 2.58 mg/l.

The results obtained from the pre-study media preparation trial (see Appendix 3) indicated that slightly higher dissolved test material concentrations were obtained from the saturated solution method of preparation in comparison to the solvent spike method of preparation. There were no significant differences in the test concentrations obtained following removal of undissolved test material by either centrifugation or filtration, nor did prolonged stirring result in higher test concentrations.

Based on the results obtained for the purposes of testing the test material was to be prepared using a saturated solution method of preparation with the aid of propeller stirring at approximately 1500 rpm for 24 hours at 21°C prior to removal of any undissolved test material by filtration through a 0.2 µm Sartorius Sartopore filter (first approximate 1 litre discarded) to give a saturated solution with a nominal concentration of 1.5 mg/l.

5.2 Range-finding Test

The cell densities and percentage inhibition of growth values from the exposure of *Desmodesmus subspicatus* to the test material during the range-finding test are given in Table 1.

The results showed no effect on growth at the test concentrations of 0.015 mg/l. However, growth was observed to be reduced at 0.15 and 1.5 mg/l.

Based on this information the test material solutions for the definitive test were prepared by stirring an excess (50 mg/l) of test material in culture medium for a period of time and then removing any undissolved test material by filtration. This saturated solution was then further diluted, as necessary, to produce the remaining test groups.

5.3 Definitive Test

Cell density values determined at each sampling time and pH values at 0 and 72 hours are given in Table 2. Daily specific growth rates for the control cultures are given in Table 3. Growth rates, yield and biomass integral values for the control and test cultures after 72 hours and percentage inhibition values are given in Table 4.

The mean cell densities versus time for the definitive test are presented in Figure 1. Percentage inhibition values are plotted against test concentration in Figure 2, Figure 3 and Figure 4, Figure 5, Figure 6 and Figure 7.

5.3.1 Validation criteria

The following data show that the cell concentration of the control cultures increased by a factor of 89 after 72 hours. This increase was in line with the OECD Guideline that states the enhancement must be at least by a factor of 16 after 72 hours.

Mean cell density of control at 0 hours : 3.47×10^3 cells per ml
Mean cell density of control at 72 hours : 3.10×10^5 cells per ml

The mean coefficient of variation for section by section specific growth rate for the control cultures was 24% and hence satisfied the validation criterion given in the OECD Guideline which states the mean must not exceed 35%.

The coefficient of variation for average specific growth rate for the control cultures over the test period (0 – 72 h) was 2% and hence satisfied the validation criterion given in the OECD Guideline which states that this must not exceed 7%.

5.3.2 Growth data

From the data given in Tables 2 and 4, it is clear that the growth rate (r), yield (y) and biomass (b) of *Desmodesmus subspicatus* (CCAP 276/20) were affected by the presence of the test material over the 72-Hour exposure period.

Accordingly the following results were determined from the data:

5.3.2.1 Inhibition of growth rate

E_rC_{10} (0 - 72 h) : 0.11 mg/l
E_rC_{20} (0 - 72 h) : 0.16 mg/l
E_rC_{50} (0 - 72 h) : 0.31 mg/l; 95% confidence limits 0.27 – 0.36 mg/l

where E_rC_x is the test concentration that reduced growth rate by x%.

Statistical analysis of the growth rate data was carried out for the control and all test concentrations using one way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955). There were no statistically significant differences between the control and 0.015 mg/l test concentration ($P \geq 0.05$), however all other test concentrations were significantly different ($P < 0.05$) and, therefore the "No Observed Effect Concentration" (NOEC) based on growth rate was 0.015 mg/l. Correspondingly the "Lowest Observed Effect Concentration" (LOEC) based on growth rate was 0.048 mg/l.

5.3.2.2 Inhibition of yield

E_yC_{10} (0 - 72 h) : 0.014 mg/l
E_yC_{20} (0 - 72 h) : 0.030 mg/l
E_yC_{50} (0 - 72 h) : 0.11 mg/l; 95% confidence limits 0.084 – 0.15 mg/l

where E_yC_x is the test concentration that reduced yield by x%.

Statistical analysis of the yield data was carried out as in Section 5.3.2.1. There were no statistically significant differences between the control and 0.015 mg/l test concentration ($P \geq 0.05$), however all other test concentrations were significantly different ($P < 0.05$) and, therefore the "No Observed Effect Concentration" (NOEC) based on yield was 0.015 mg/l. Correspondingly the "Lowest Observed Effect Concentration" (LOEC) based on yield was 0.048 mg/l.

5.3.2.3 Inhibition of biomass integral

E_bC_{10} (0 - 72 h) : 0.017 mg/l
E_bC_{20} (0 - 72 h) : 0.037 mg/l
E_bC_{50} (0 - 72 h) : 0.13 mg/l; 95% confidence limits 0.10 – 0.18 mg/l

where E_bC_x is the test concentration that reduced biomass integral (area under the growth curve) by x%.

Statistical analysis of the biomass integral data was carried out as in Section 5.3.2.1. There were no statistically significant differences between the control and 0.015 mg/l test concentration ($P \geq 0.05$), however all other test concentrations were significantly different ($P < 0.05$) and, therefore the "No Observed Effect Concentration" (NOEC) based on biomass integral was 0.015 mg/l. Correspondingly the "Lowest Observed Effect Concentration" (LOEC) based on biomass integral was 0.048 mg/l.

5.3.3 Observations on cultures

All test and control cultures were inspected microscopically at 72 hours. There were no abnormalities detected in any of the control or test cultures at 72 hours.

5.3.4 Observations on test material solubility

At the start of the test all control and test cultures were observed to be clear colourless solutions. After the 72-Hour test period all control, 0.015, 0.048 and 0.15 mg/l test cultures were observed to be green dispersions whilst the 0.48 and 1.5 mg/l test cultures were observed to be clear colourless solutions.

5.3.5 Physico-chemical measurements

The pH values of each test and control flask are given in Table 2. Temperature was maintained at 24 ± 1°C throughout the test.

The pH values of the control cultures (see Table 2) were observed to increase from pH 7.3 at 0 hours to pH 7.5 at 72 hours. The pH deviation in the control cultures was less than 1.5 pH units after 72 hours and therefore was within the limits given in the Test Guidelines.

5.3.6 Verification of test concentrations

Analysis of the test preparations at 0 hours (see Appendix 3) showed measured test concentrations to range from 84% to 121% of nominal. Analysis of the test preparations at 72 hours (see Appendix 3) showed a decline in measured test concentrations in the range of less than 1% of

nominal to 71% of nominal. This decline was inline with the stability analyses conducted which indicated that the test material was unstable in culture medium over the test duration particularly at the lower test concentrations employed. A further decline in excess of that seen in the stability analyses was considered to be due to possible adsorption of the test material to the algal cells present particularly at the lower test concentrations employed. This effect was considered to be due to there being greater numbers of algal cells in the lower concentrations and hence greater surface area for adsorption to occur. Whilst no immediate adsorption was observed in the recovery analyses conducted in the presence of algal cells this does not preclude long-term adsorption over the test period. Adsorption was not a factor in the stability analyses as no algal cells were present.

Current regulatory advice is that in cases where a decline in measured concentrations is observed, geometric mean measured concentrations should be used for calculating EC_{50} values. It was therefore considered justifiable to base the results on the geometric mean measured test concentrations in order to give a "worst case" analysis of the data. The geometric mean measured test concentrations were determined to be:

Nominal Test Concentration (mg/l)	Geometric Mean Measured Test Concentration (mg/l)	Expressed as a % of the Nominal Test Concentration
0.015	0.0018	12
0.048	0.0045	9
0.15	0.010	7
0.48	0.43	90
1.5	1.4	93

The following results were determined from the data based on the geometric mean measured test concentrations.

Growth rate

E_rC_{10} (0 - 72 h) : 0.0044 mg/l
E_rC_{20} (0 - 72 h) : 0.019 mg/l
E_rC_{50} (0 - 72 h) : 0.15 mg/l; 95% confidence limits 0.10 – 0.23 mg/l
No Observed Effect Concentration (NOEC) = 0.0018 mg/l
Lowest Observed Effect Concentration (LOEC) = 0.0045 mg/l

Yield

E_yC_{10} (0 - 72 h) : 0.0011 mg/l
E_yC_{20} (0 - 72 h) : 0.0025 mg/l
E_yC_{50} (0 - 72 h) : 0.010 mg/l; 95% confidence limits 0.0070 – 0.014 mg/l
No Observed Effect Concentration (NOEC) = 0.0018 mg/l
Lowest Observed Effect Concentration (LOEC) = 0.0045 mg/l

Biomass integral

E_bC_{10} (0 - 72 h) : 0.0011 mg/l
E_bC_{20} (0 - 72 h) : 0.0029 mg/l
E_bC_{50} (0 - 72 h) : 0.014 mg/l; 95% confidence limits 0.010 – 0.021 mg/l
No Observed Effect Concentration (NOEC) = 0.0018 mg/l
Lowest Observed Effect Concentration (LOEC) = 0.0045 mg/l

The use of the geometric mean measured test concentrations in the calculation of the EC_{50} and NOEC values had a significant effect on the outcome of the study.

5.4 Positive Control

The cell densities from exposure of *Desmodesmus subspicatus* (CCAP 276/20) to the reference material during the positive control (Safepharm Laboratories Project No: 0039/0941) are given in Table 5 and Figure 8. Daily specific growth rates for the control cultures are given in Table 6 whilst growth rates, yield and biomass integral values are given in Table 7. Percentage inhibition values are plotted against test concentration in Figure 9, Figure 10 and Figure 11.

Accordingly the following results were determined from the data:

E_rC_{50} (0 - 72 h) : 0.49 mg/l; 95% confidence limits 0.43 - 0.55 mg/l
E_yC_{50} (0 - 72 h) : 0.22 mg/l; 95% confidence limits 0.19 - 0.24 mg/l
E_bC_{50} (0 - 72 h) : 0.23 mg/l; 95% confidence limits 0.21 - 0.27 mg/l

No Observed Effect Concentration (NOEC) based on growth rate : 0.125 mg/l
No Observed Effect Concentration (NOEC) based on yield : 0.0625 mg/l
No Observed Effect Concentration (NOEC) based on biomass integral : 0.0625 mg/l

Lowest Observed Effect Concentration (LOEC) based on growth rate	: 0.25 mg/l
Lowest Observed Effect Concentration (LOEC) based on yield	: 0.125 mg/l
Lowest Observed Effect Concentration (LOEC) based on biomass integral	: 0.125 mg/l

The results from the positive control with potassium dichromate were within the normal range for this reference material.

6. CONCLUSION

The effect of the test material on the growth of *Desmodesmus subspicatus* has been investigated over a 72-Hour period and gave an E_rC_{50} (0 - 72 h) of 0.31 mg/l; 95% confidence limits 0.27 – 0.36 mg/l, an E_yC_{50} (0 - 72 h) of 0.11 mg/l; 95% confidence limits 0.084 – 0.15 mg/l, and an E_bC_{50} (0 - 72 h) of 0.13 mg/l; 95% confidence limits 0.10 – 0.18 mg/l. The Lowest Observed Effect Concentration based on growth rate, yield and biomass integral was 0.048 mg/l, and the No Observed Effect Concentration was 0.015 mg/l.

Based on the geometric mean measured test concentrations the E_rC_{50} (0 - 72 h) value was 0.15 mg/l; 95% confidence limits 0.10 – 0.23 mg/l, the E_yC_{50} (0 - 72 h) value was 0.010 mg/l; 95% confidence limits 0.0070 – 0.014 mg/l and the E_bC_{50} (0 - 72 h) value was 0.014 mg/l; 95% confidence limits 0.010 – 0.021 mg/l. The Lowest Observed Effect Concentration based on growth rate, yield and biomass integral was 0.0045 mg/l, and the No Observed Effect Concentration was 0.0018 mg/l.

7. REFERENCES

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ALGAL GROWTH INHIBITION TEST

Table 1 Cell Densities and Percentage Inhibition of Growth from the Range-finding Test

Nominal Concentration (mg/l)*		Cell Densities** (cells per ml)		Inhibition Values (%)	
		0 Hours	72 Hours	Growth Rate	Yield/Biomass Integral
Control	R_1	6.70E+03	5.94E+05		
	R_2	4.34E+03	5.77E+05	-	-
	Mean	5.52E+03	5.86E+05		
0.015	R_1	6.84E+03	5.13E+05		
	R_2	5.20E+03	5.52E+05	5	9
	Mean	6.02E+03	5.32E+05		
0.15	R_1	5.69E+03	2.15E+05		
	R_2	4.76E+03	2.16E+05	20	64
	Mean	5.22E+03	2.16E+05		
1.5	R_1	5.27E+03	1.34E+04		
	R_2	4.64E+03	1.02E+04	82	99
	Mean	4.96E+03	1.18E+04		

* Concentrations based on analysis of a saturated solution prepared in an identical manner during the media preparation trial.

** Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.

R_1 and R_2 = Replicates 1 and 2

ALGAL GROWTH INHIBITION TEST

Table 2 Cell Densities and pH Values in the Definitive Test

Nominal Concentration (mg/l)		pH	Cell Densities* (cells per ml)				pH
		0 h	0 h	24 h	48 h	72 h	72 h
Control	R_1	7.3	3.72E+03	1.52E+04	5.43E+04	3.18E+05	7.5
	R_2	7.3	3.13E+03	1.84E+04	6.33E+04	2.83E+05	7.5
	R_3	7.3	4.16E+03	1.55E+04	5.70E+04	2.84E+05	7.5
	R_4	7.3	3.29E+03	1.18E+04	3.99E+04	3.46E+05	7.5
	R_5	7.3	3.48E+03	1.10E+04	4.62E+04	3.13E+05	7.5
	R_6	7.3	3.02E+03	1.36E+04	4.14E+04	3.15E+05	7.5
	Mean		3.47E+03	1.42E+04	5.03E+04	3.10E+05	
0.015	R_1	7.3	3.82E+03	1.39E+04	4.77E+04	2.52E+05	7.6
	R_2	7.3	3.91E+03	1.42E+04	4.98E+04	3.10E+05	7.6
	R_3	7.3	2.99E+03	1.63E+04	5.32E+04	2.26E+05	7.6
	Mean		3.58E+03	1.48E+04	5.02E+04	2.63E+05	
0.048	R_1	7.3	3.84E+03	1.15E+04	4.60E+04	2.47E+05	7.6
	R_2	7.3	3.98E+03	1.45E+04	4.27E+04	1.66E+05	7.6
	R_3	7.3	3.41E+03	1.27E+04	3.42E+04	1.75E+05	7.6
	Mean		3.74E+03	1.29E+04	4.10E+04	1.96E+05	
0.15	R_1	7.3	3.88E+03	1.24E+04	3.74E+04	1.57E+05	7.6
	R_2	7.3	4.42E+03	1.35E+04	3.72E+04	1.67E+05	7.6
	R_3	7.3	3.62E+03	1.13E+04	3.88E+04	1.93E+05	7.5
	Mean		3.97E+03	1.24E+04	3.78E+04	1.72E+05	
0.48	R_1	7.3	3.60E+03	4.63E+03	3.66E+03	1.15E+04	7.4
	R_2	7.3	4.99E+03	4.98E+03	3.26E+03	1.37E+04	7.4
	R_3	7.3	5.55E+03	4.38E+03	3.65E+03	9.57E+03	7.4
	Mean		4.71E+03	4.67E+03	3.52E+03	1.16E+04	
1.5	R_1	7.2	4.72E+03	3.59E+03	2.35E+03	2.21E+03	7.4
	R_2	7.2	4.01E+03	3.71E+03	2.22E+03	1.85E+03	7.4
	R_3	7.2	4.80E+03	3.57E+03	2.84E+03	2.58E+03	7.4
	Mean		4.51E+03	3.62E+03	2.47E+03	2.21E+03	

* Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.

R_1 - R_6 = Replicates 1 to 6

Table 3 Daily Specific Growth Rates for the Control Cultures in the Definitive Test

		Daily Specific Growth Rate		
		Day 0 - 1	Day 1 - 2	Day 2 - 3
Control	R_1	0.056	0.053	0.074
	R_2	0.064	0.051	0.062
	R_3	0.056	0.054	0.067
	R_4	0.045	0.051	0.090
	R_5	0.042	0.060	0.080
	R_6	0.051	0.046	0.085
	Mean	0.052	0.053	0.076

R_1 - R_6 = Replicates 1 to 6

Table 4 Inhibition of Growth Rate, Yield and Biomass Integral in the Definitive Test

Nominal Concentration (mg/l)		Growth Rate (cells/ml/hour)		Yield (cells/ml)		Biomass Integral	
		0 – 72 h	% Inhibition	0 – 72 h	% Inhibition*	0 – 72 h	% Inhibition
Control	R_1	0.061		3.15E+05		5.25E+06	
	R_2	0.059		2.80E+05		5.11E+06	
	R_3	0.059		2.79E+05		4.90E+06	
	R_4	0.062	-	3.43E+05	-	5.16E+06	-
	R_5	0.061		3.09E+05		4.89E+06	
	R_6	0.061		3.12E+05		4.86E+06	
	Mean	0.061		3.06E+05		5.03E+06	
	SD	0.001		2.40E+04		1.65E+05	
0.015	R_1	0.058	5	2.48E+05		4.26E+06	15
	R_2	0.060	2	3.06E+05		5.01E+06	0
	R_3	0.056	8	2.23E+05		4.14E+06	18
	Mean	0.058	5	2.59E+05	15	4.47E+06	11
	SD	0.002		4.24E+04		4.74E+05	
0.048	R_1	0.057	7	2.43E+05		4.10E+06	18
	R_2	0.052	15	1.62E+05		3.12E+06	38
	R_3	0.052	15	1.71E+05		2.98E+06	41
	Mean	0.054	12	1.92E+05	37	3.40E+06	32
	SD	0.003		4.44E+04		6.11E+05	
0.15	R_1	0.051	16	1.54E+05		2.84E+06	43
	R_2	0.052	15	1.63E+05		2.98E+06	41
	R_3	0.054	11	1.89E+05		3.28E+06	35
	Mean	0.052	14	1.68E+05	45	3.03E+06	40
	SD	0.002		1.85E+04		2.21E+05	
0.48	R_1	0.015	75	7.94E+03		9.77E+04	98
	R_2	0.017	72	8.71E+03		1.22E+05	98
	R_3	0.012	80	4.02E+03		6.77E+04	99
	Mean	0.015	76	6.89E+03	98	9.59E+04	98
	SD	0.003		2.52E+03		2.73E+04	
1.5	R_1	-0.008	113	-2.51E+03		-7.10E+04	101
	R_2	-0.011	118	-2.16E+03		-7.56E+04	102
	R_3	-0.006	110	-2.22E+03		-5.52E+04	101
	Mean	-0.008	114	-2.30E+03	101	-6.73E+04	101
	SD	0.003		1.89E+02		1.07E+04	

* In accordance with the OECD test guideline only the mean value for yield for each test concentration is calculated
$R_1 – R_6$ = Replicates 1 to 6
SD = Standard Deviation

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Table 5 Cell Densities and pH Values in the Positive Control

Nominal Concentration (mg/l)		pH	Cell Densities* (cells per ml)				pH
		0 h	0 h	28 h	52 h	72 h	72 h
Control	R_1	7.3	4.17E+03	1.70E+04	9.72E+04	5.36E+05	7.7
	R_2	7.3	4.36E+03	1.69E+04	1.07E+05	4.72E+05	7.7
	R_3	7.3	4.23E+03	1.73E+04	8.95E+04	4.34E+05	7.7
	R_4	7.3	4.26E+03	1.75E+04	1.18E+05	5.19E+05	7.8
	R_5	7.3	4.14E+03	1.79E+04	1.07E+05	4.90E+05	7.8
	R_6	7.3	4.08E+03	1.65E+04	7.85E+04	4.63E+05	7.7
	Mean		4.20E+03	1.72E+04	9.96E+04	4.86E+05	
0.0625	R_1	7.3	4.22E+03	2.04E+04	8.90E+04	5.69E+05	7.7
	R_2	7.3	4.15E+03	1.87E+04	9.39E+04	4.95E+05	7.7
	R_3	7.3	4.28E+03	1.64E+04	9.56E+04	5.29E+05	7.7
	Mean		4.22E+03	1.85E+04	9.28E+04	5.31E+05	
0.125	R_1	7.3	3.88E+03	1.51E+04	9.00E+04	4.07E+05	7.8
	R_2	7.3	4.11E+03	1.58E+04	9.07E+04	3.56E+05	7.7
	R_3	7.3	4.27E+03	1.64E+04	9.34E+04	3.70E+05	7.7
	Mean		4.09E+03	1.57E+04	9.13E+04	3.78E+05	
0.25	R_1	7.3	4.08E+03	1.01E+04	6.46E+04	1.98E+05	7.7
	R_2	7.3	4.03E+03	1.02E+04	5.37E+04	2.11E+05	7.7
	R_3	7.3	4.28E+03	1.08E+04	3.98E+04	2.14E+05	7.7
	Mean		4.13E+03	1.04E+04	5.27E+04	2.08E+05	
0.50	R_1	7.3	4.32E+03	9.58E+03	2.19E+04	5.41E+04	7.6
	R_2	7.3	4.48E+03	1.20E+04	2.92E+04	4.46E+04	7.6
	R_3	7.3	4.24E+03	1.06E+04	2.01E+04	2.78E+04	7.6
	Mean		4.35E+03	1.07E+04	2.37E+04	4.22E+04	
1.0	R_1	7.2	4.10E+03	8.28E+03	8.79E+03	1.15E+04	7.5
	R_2	7.2	4.12E+03	7.62E+03	6.37E+03	9.70E+03	7.5
	R_3	7.2	4.01E+03	6.97E+03	5.69E+03	8.80E+03	7.5
	Mean		4.07E+03	7.62E+03	6.95E+03	9.99E+03	

* Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.

R_1 - R_6 = Replicates 1 to 6

Table 6 Daily Specific Growth Rates for the Control Cultures in the Positive Control

		Daily Specific Growth Rate		
		Day 0 - 1	Day 1 - 2	Day 2 - 3
Control	R_1	0.052	0.073	0.085
	R_2	0.051	0.077	0.074
	R_3	0.052	0.068	0.079
	R_4	0.053	0.080	0.074
	R_5	0.054	0.074	0.076
	R_6	0.051	0.065	0.089
	Mean	0.052	0.073	0.080

R_1 - R_6 = Replicates 1 to 6

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Table 7 Inhibition of Growth Rate, Yield and Biomass Integral in the Positive Control

Nominal Concentration (mg/l)		Growth Rate (cells/ml/hour)		Yield (cells/ml)		Biomass Integral	
		0 – 72 h	% Inhibition	0 – 72 h	% Inhibition*	0 – 72 h	% Inhibition
Control	R_1	0.068		5.32E+05		7.71E+06	
	R_2	0.066		4.68E+05		7.29E+06	
	R_3	0.065		4.30E+05		6.53E+06	
	R_4	0.068	-	5.15E+05	-	8.02E+06	-
	R_5	0.067		4.86E+05		7.49E+06	
	R_6	0.066		4.59E+05		6.55E+06	
	Mean	0.067		4.82E+05		7.27E+06	
	SD	0.001		3.75E+04		6.09E+05	
0.0625	R_1	0.069	[3]	5.65E+05		7.95E+06	[9]
	R_2	0.067	0	4.91E+05		7.27E+06	0
	R_3	0.068	[1]	5.24E+05		7.58E+06	[4]
	Mean	0.068	[1]	5.27E+05	[9]	7.60E+06	[4]
	SD	0.001		3.70E+04		3.37E+05	
0.125	R_1	0.064	4	4.03E+05		6.21E+06	15
	R_2	0.062	7	3.52E+05		5.73E+06	21
	R_3	0.063	6	3.65E+05		5.95E+06	18
	Mean	0.063	6	3.73E+05	22	5.96E+06	18
	SD	0.001		2.68E+04		2.41E+05	
0.25	R_1	0.054	19	1.94E+05		3.43E+06	53
	R_2	0.055	18	2.07E+05		3.32E+06	54
	R_3	0.055	18	2.10E+05		3.07E+06	58
	Mean	0.055	18	2.04E+05	58	3.28E+06	55
	SD	0.001		8.43E+03		1.87E+05	
0.50	R_1	0.036	46	4.97E+04		1.04E+06	86
	R_2	0.033	51	4.01E+04		1.17E+06	84
	R_3	0.027	60	2.36E+04		7.65E+05	89
	Mean	0.032	52	3.78E+04	92	9.91E+05	86
	SD	0.005		1.32E+04		2.06E+05	
1.0	R_1	0.015	78	7.37E+03		2.91E+05	96
	R_2	0.012	82	5.58E+03		2.03E+05	97
	R_3	0.011	84	4.79E+03		1.62E+05	98
	Mean	0.013	81	5.91E+03	99	2.19E+05	97
	SD	0.002		1.32E+03		6.60E+04	

* In accordance with the OECD test guideline only the mean value for yield for each test concentration is calculated
$R_1 – R_6$ = Replicates 1 to 6
SD = Standard Deviation
[Increase in growth as compared to controls]

ALGAL GROWTH INHIBITION TEST

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Figure 1 Mean Cell Densities v Time for the Definitive Test

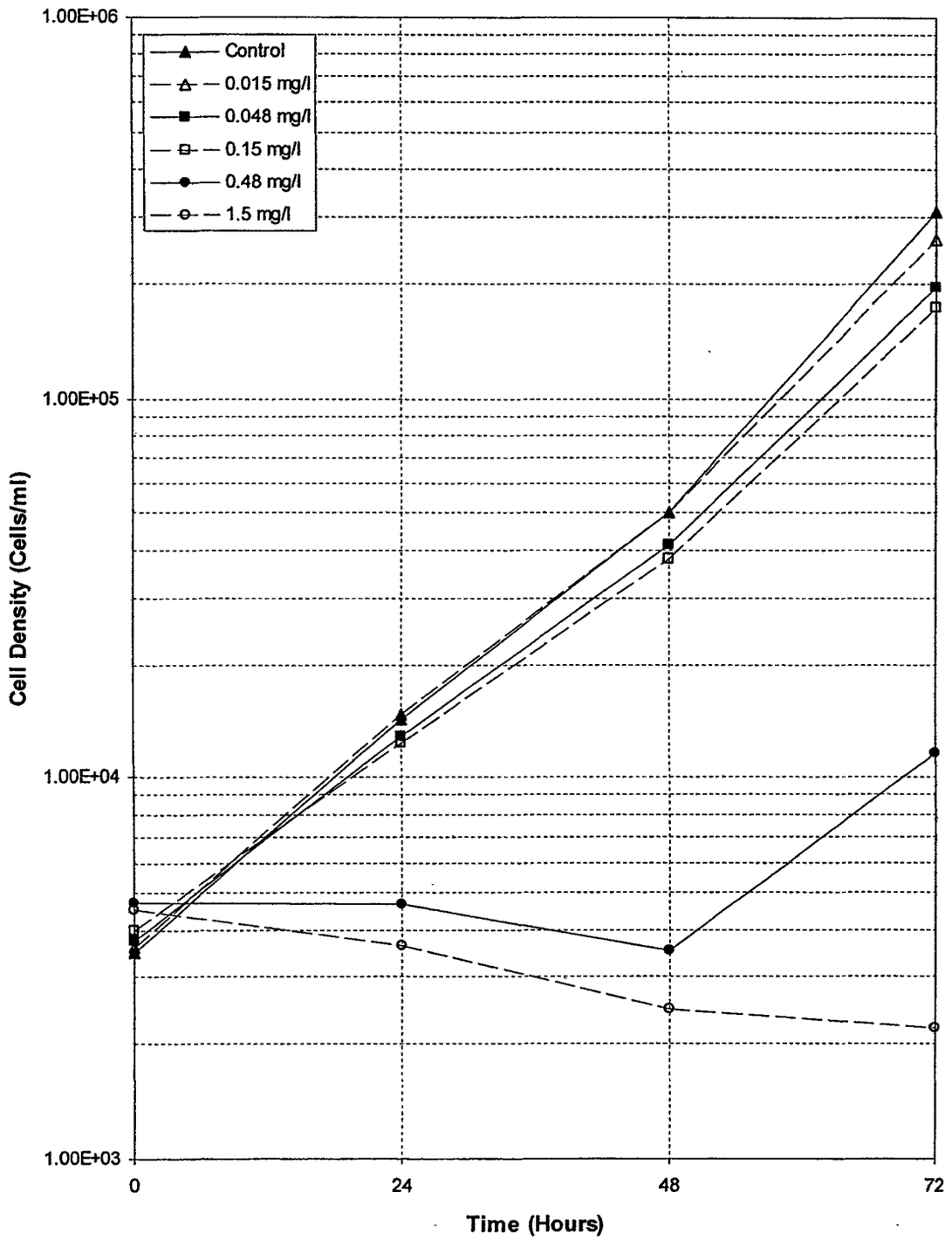
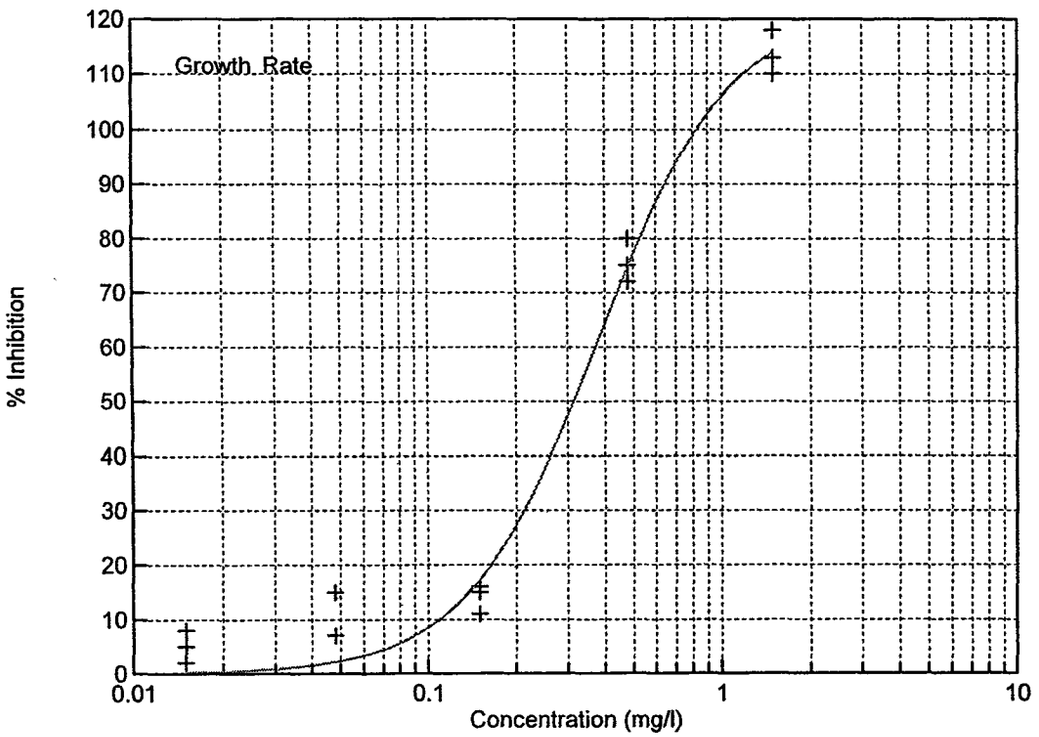
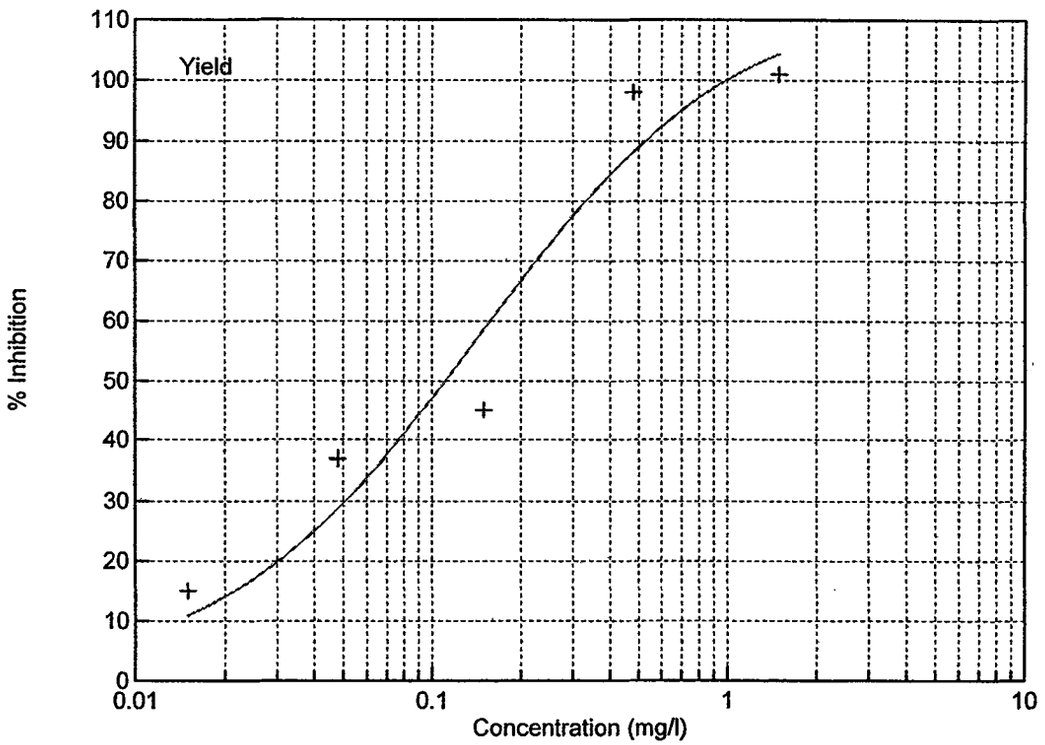


Figure 2 Inhibition of Growth Rate Based on Nominal Test Concentrations



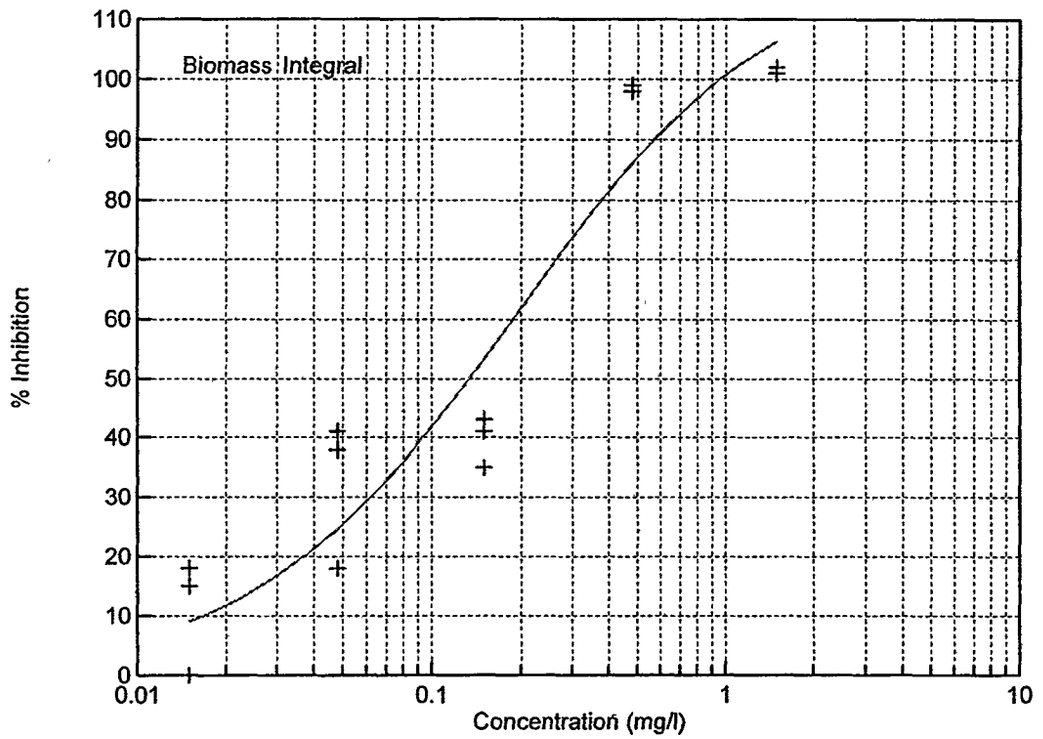
ALGAL GROWTH INHIBITION TEST

Figure 3 Inhibition of Yield Based on Nominal Test Concentrations



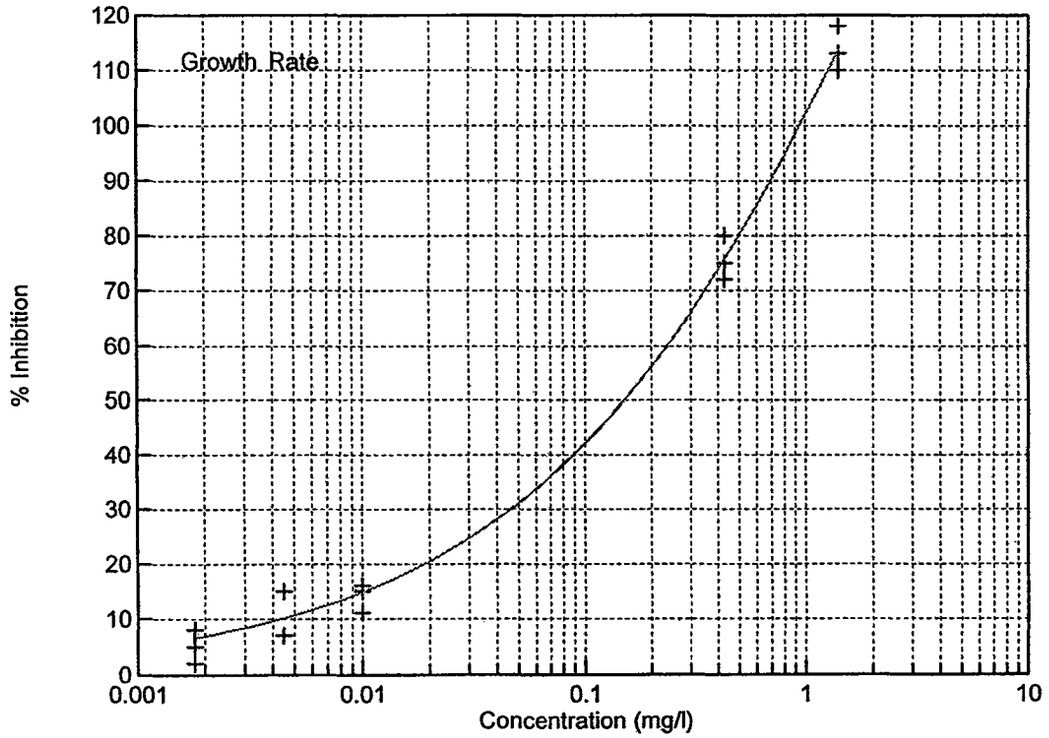
ALGAL GROWTH INHIBITION TEST

Figure 4 Inhibition of Biomass Integral Based on Nominal Test Concentrations



ALGAL GROWTH INHIBITION TEST

Figure 5 Inhibition of Growth Rate Based on Geometric Mean Measured Test Concentrations



ALGAL GROWTH INHIBITION TEST

Figure 6 **Inhibition of Yield Based on Geometric Mean Measured Test Concentrations**

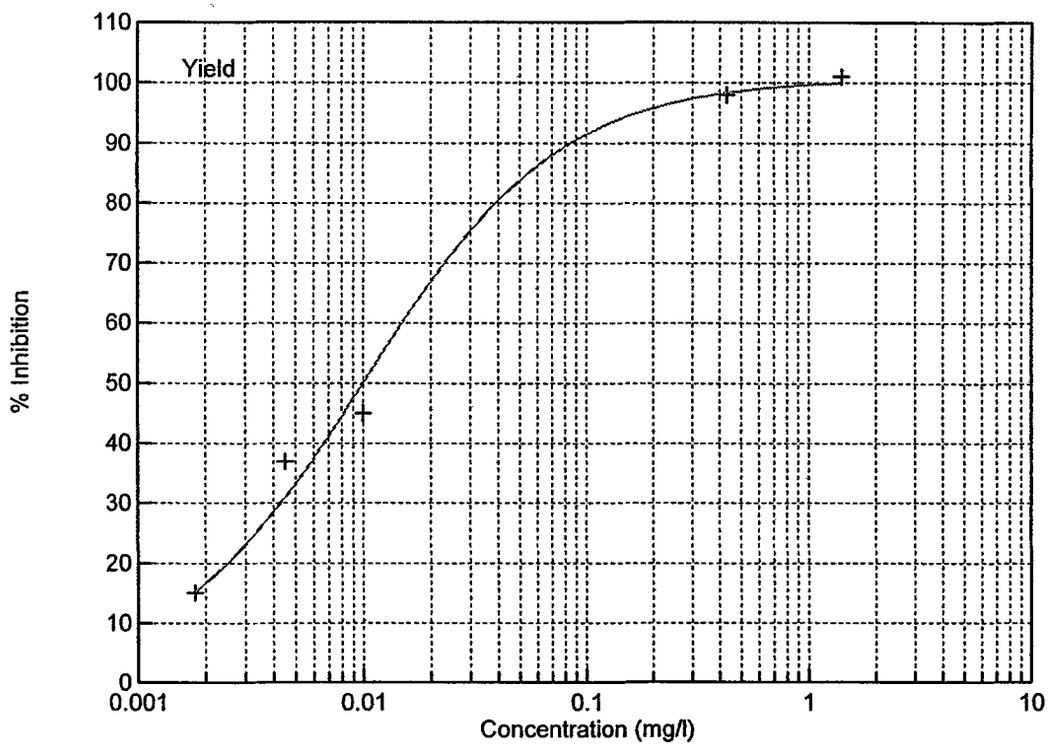


Figure 7 **Inhibition of Biomass Integral Based on Geometric Mean Measured Test Concentrations**

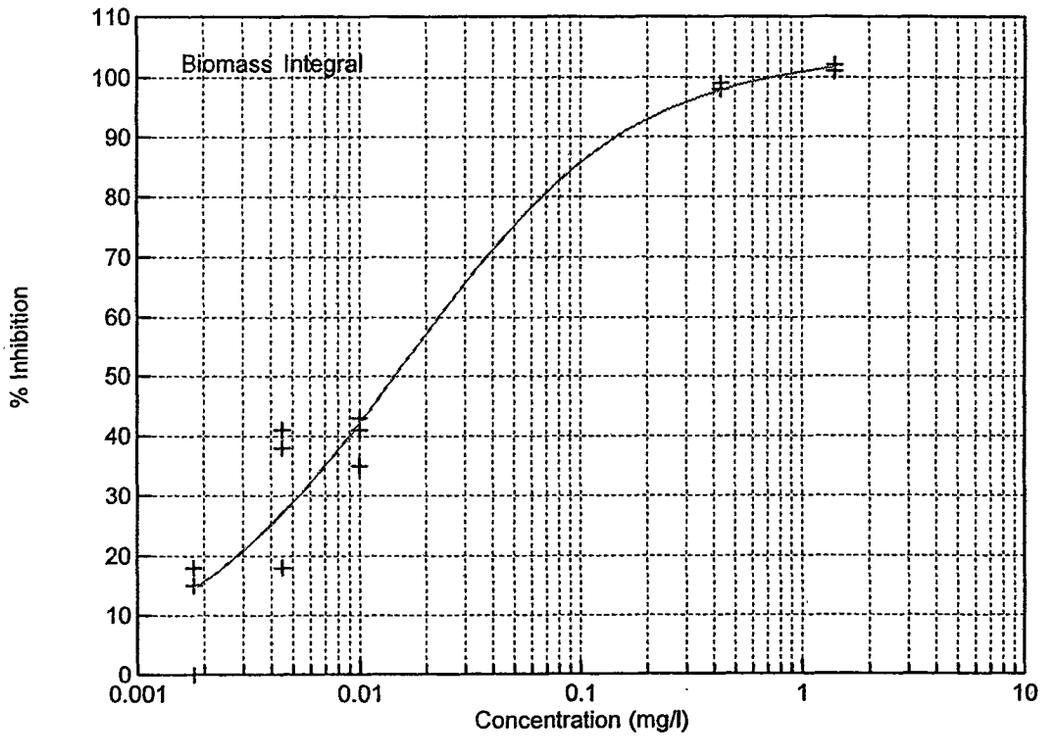


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Figure 8 Mean Cell Densities v Time for the Positive Control

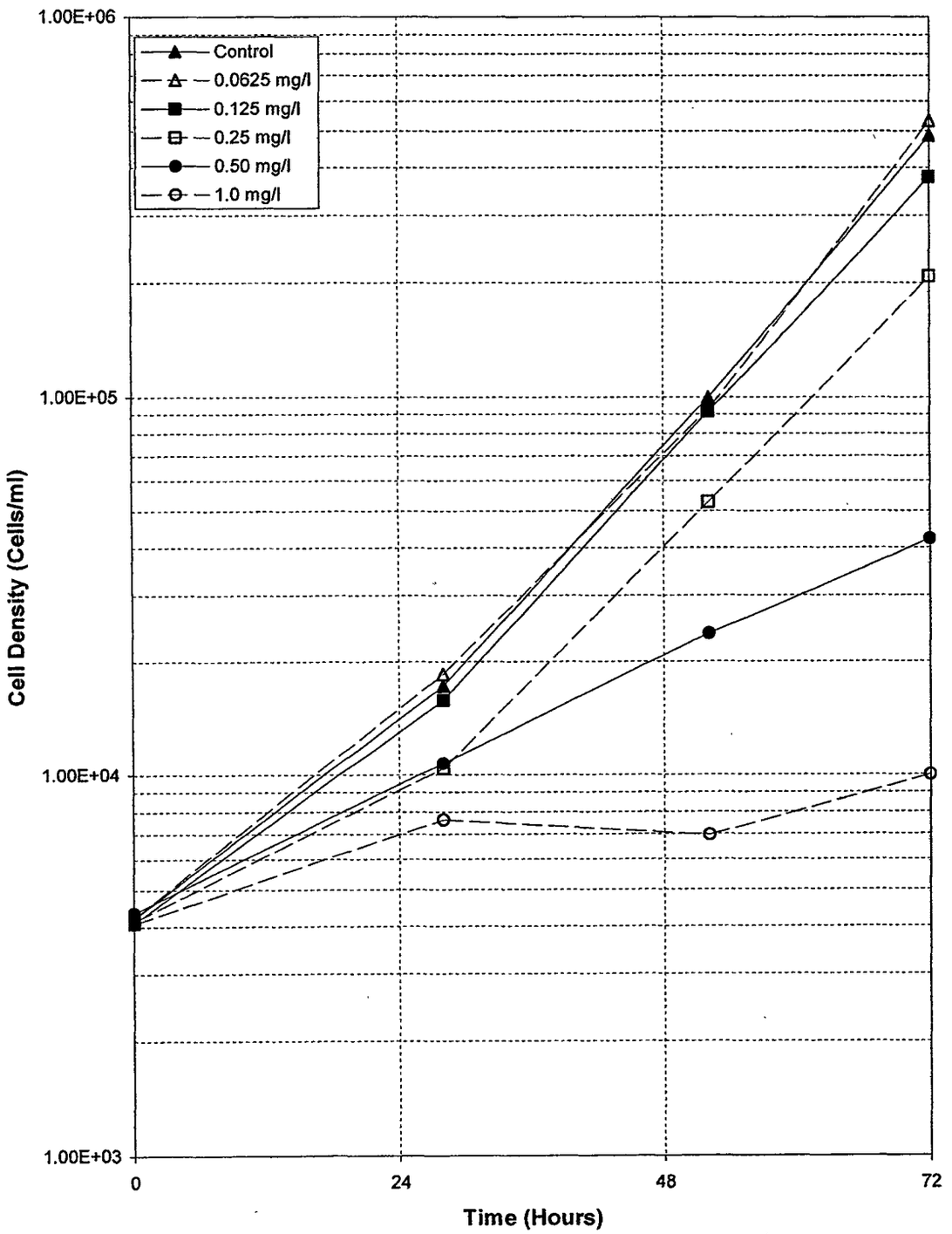

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Figure 9 Inhibition of Growth Rate for the Positive Control

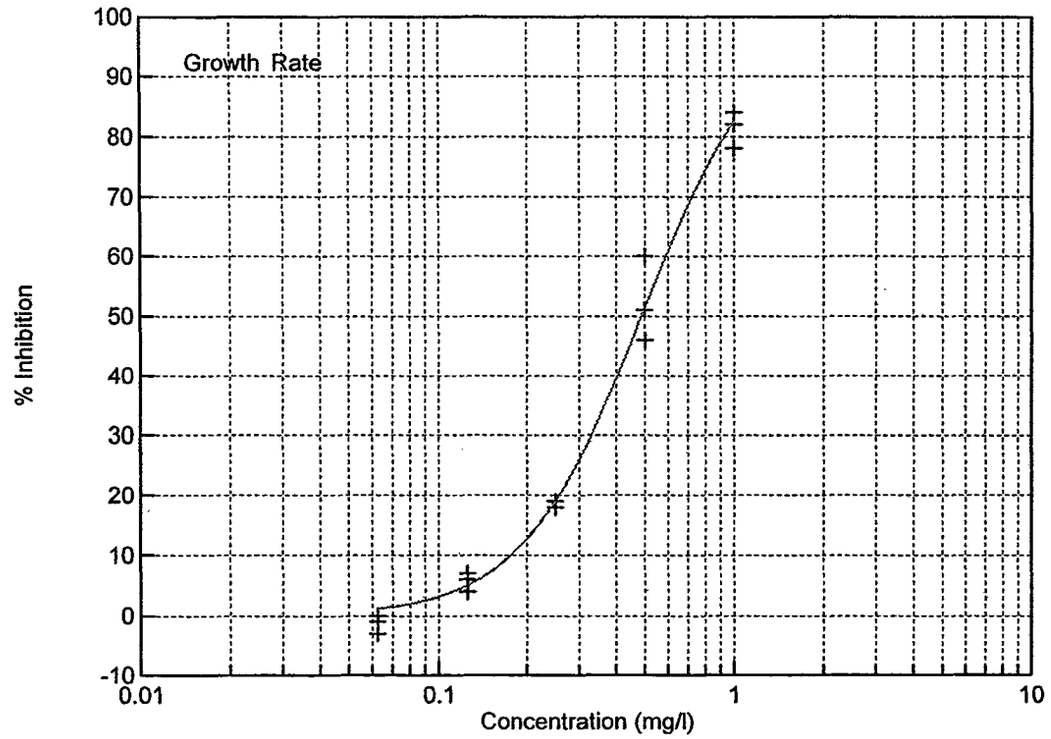

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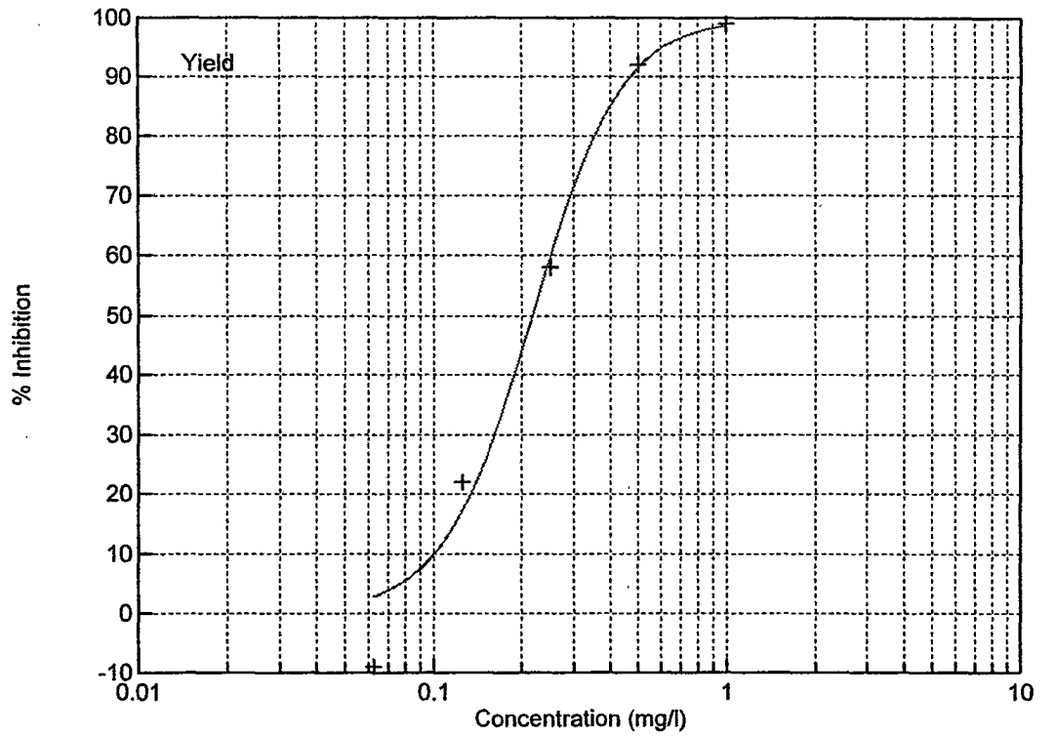
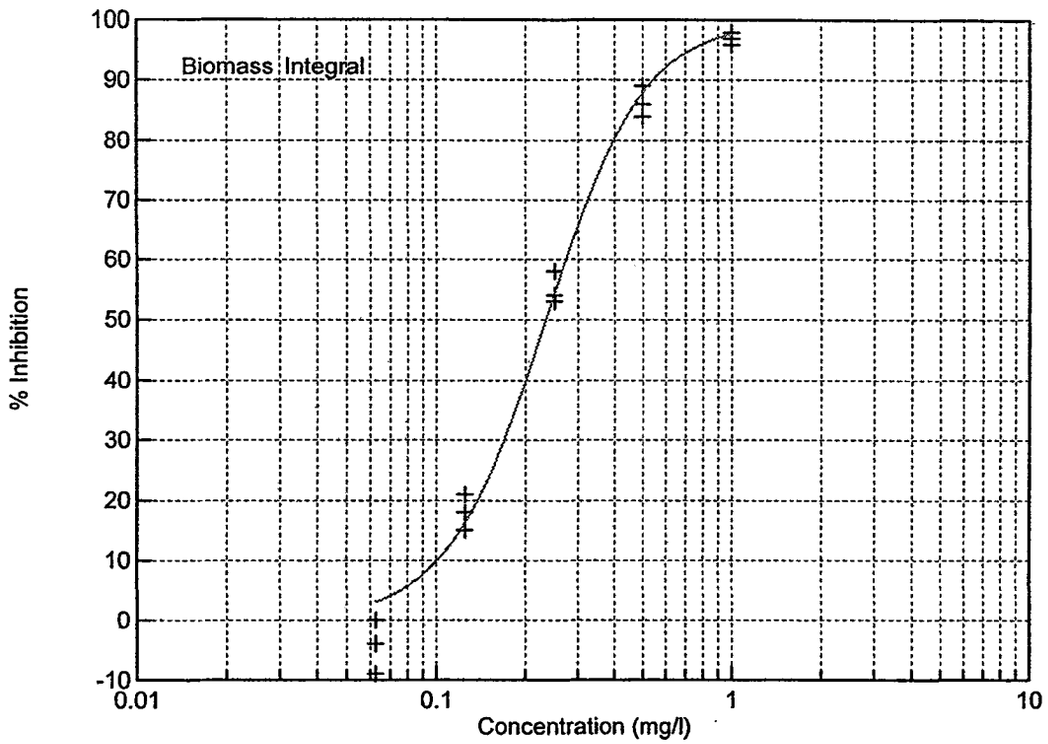
Figure 10 Inhibition of Yield for the Positive Control



ALGAL GROWTH INHIBITION TEST

Figure 11 Inhibition of Biomass Integral for the Positive Control



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Appendix 1 Certificate of Analysis

CERTIFICATE OF ANALYSIS

Date : 02-20-2007

PARAMETER	RESULT
^{1}HNMR	97.0 %
Appearance	Light yellow solid
Melting point	32-33.6 °C

ALGAL GROWTH INHIBITION TEST

Appendix 2 Culture Medium

$NaNO_3$	25.5	mg/l
$MgCl_2.6H_2O$	12.164	mg/l
$CaCl_2.2H_2O$	4.41	mg/l
$MgSO_4.7H_2O$	14.7	mg/l
K_2HPO_4	1.044	mg/l
$NaHCO_3$	15.0	mg/l
H_3BO_3	0.1855	mg/l
$MnCl_2.4H_2O$	0.415	mg/l
$ZnCl_2$	0.00327	mg/l
$FeCl_3.6H_2O$	0.159	mg/l
$CoCl_2.6H_2O$	0.00143	mg/l
$Na_2MoO_4.2H_2O$	0.00726	mg/l
$CuCl_2.2H_2O$	0.000012	mg/l
$Na_2EDTA.2H_2O$	0.30	mg/l
$Na_2SeO_3.5H_2O$	0.000010	mg/l

The culture medium was prepared using reverse osmosis purified deionised water* and the pH adjusted to 7.5 ± 0.1 with 0.1N NaOH or HCl.

* Elga Optima 15+ or Elga Purelab Option R-15 BP

Appendix 3 Verification of Test Concentrations

1. METHOD OF ANALYSIS

1.1 Introduction

The test material concentration in the test samples was determined by high performance liquid chromatography (HPLC) using an external standard. The test material gave a chromatographic profile consisting of a single peak.

The method was developed by the Department of Analytical Services, Safepharm Laboratories Limited.

1.2 Sample Preparation

1.2.1 Pre-Study Media Preparation Trial, Solvent Spike Preparation, 0 Hours

A volume of test sample (50 ml) was extracted with dichloromethane (3 x 50 ml). The extracts were filtered through anhydrous sodium sulphate. The combined extracts were evaporated to dryness and the residue re-dissolved in methanol (5 ml) to give a final theoretical concentration of 20 mg/l.

1.2.2 All Other Sample Preparations

A Strata X solid phase extraction (SPE) cartridge was sequentially pre-conditioned with methanol and water*. A volume of test sample was eluted through the cartridge and the cartridge dried. The test material was eluted from the cartridge with methanol and made to volume to give a final theoretical concentration between approximately 2 and 20 mg/l.

1.3 Standards

Standard solutions of test material were prepared in methanol at a nominal concentration of 10 mg/l.

---

* Prepared by ELGA Purelab Option R-15 water purification

Appendix 3 (continued) Verification of Test Concentrations

1.4 Procedure

The standards and samples were analysed by HPLC using the following conditions:

HPLC System	:	Agilent Technologies 1050 or 1100, incorporating autosampler and workstation
Column	:	Phenosphere Next Phenyl, 5 µ, (250 x 4.6 mm id)
Column temperature	:	ambient
Mobile phase	:	methanol:water* (75:25, v/v)
Flow rate	:	1 ml/min
UV/Vis detector wavelength	:	254 nm
Injection volume	:	25 µl
Retention time	:	approximately 7 minutes

2. PRE-STUDY MEDIA PREPARATION TRIAL

2.1 Saturated Solution Preparation

An amount of test material (550 mg) was dispersed, in duplicate, in 11 litres of culture medium to give initial test material dispersions of 50 mg/l. These were stirred using a propeller stirrer at approximately 1500 rpm at approximately 21°C for periods of 24 and 48 hours.

Samples were taken for analysis following removal of any undissolved test material by centrifugation at 10000 or 40000 *g* for 30 minutes or following filtration through 0.2 µm Sartorius Sartopore filters with the first 1 or 2 litres being discarded.

Stirring Period and Treatment	Concentration Found (mg/l)
24 Hours Control	<LOQ
24 Hours Centrifuged 10000 *g*	1.72
24 Hours Centrifuged 40000 *g*	1.37
24 Hours Filtered 1 litre discarded	1.53
24 Hours Filtered 2 litres discarded	1.59

* Prepared by ELGA Purelab Option R-15 water purification
LOQ = Limit of quantitation

Appendix 3 (continued) Verification of Test Concentrations

Stirring Period and Treatment	Concentration Found (mg/l)
48 Hours Control	<LOQ
48 Hours Centrifuged 10000 *g*	1.14
48 Hours Centrifuged 40000 *g*	1.47
48 Hours Filtered 1 litre discarded	1.06
48 Hours Filtered 2 litres discarded	1.20

2.2 Solvent Spike Preparation

An amount of test material (200 mg) was dissolved in 10 ml of dimethylformamide to give a 200 mg/10 ml solvent stock solution. An aliquot (1.0 ml) of this solvent stock solution was dispersed in 10 litres of culture medium with the aid of magnetic stirring for approximately 10 minutes to give a nominal test concentration of 2.0 mg/l. This test concentration was then stirred by magnetic stirrer at 21°C with samples taken for analysis initially and after stirring for periods of 24 or 48 hours.

Samples were analysed untreated, following centrifugation at 10000 or 40000 *g* for 30 minutes or following filtration through 0.2 µm Gelman Acrocap filters with the first 100 or 500 ml being discarded.

Stirring Period and Treatment	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
0 Hours Control	Control	<LOQ	-
0 Hours Untreated	2.0	1.53	77
0 Hours Centrifuged 10000 *g*	2.0	0.726	36
0 Hours Centrifuged 40000 *g*	2.0	0.713	36
0 Hours Filtered 100 ml discarded	2.0	0.953	48
0 Hours Filtered 500 ml discarded	2.0	1.03	51

LOQ = Limit of quantitation

ALGAL GROWTH INHIBITION TEST

Appendix 3 (continued) Verification of Test Concentrations

Stirring Period and Treatment	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
24 Hours Control	Control	<LOQ	-
24 Hours Untreated	2.0	1.77	89
24 Hours Centrifuged 10000 *g*	2.0	1.01	51
24 Hours Centrifuged 40000 *g*	2.0	0.923	46
24 Hours Filtered 100 ml discarded	2.0	1.56	78
24 Hours Filtered 500 ml discarded	2.0	1.59	80
48 Hours Control	Control	<LOQ	-
48 Hours Untreated	2.0	1.61	81
48 Hours Centrifuged 10000 *g*	2.0	0.962	48
48 Hours Centrifuged 40000 *g*	2.0	0.953	48
48 Hours Filtered 100 ml discarded	2.0	1.48	74
48 Hours Filtered 500 ml discarded	2.0	1.48	74

3. VALIDATION

3.1 Linearity

A range of standard solutions covering 0.10 to 51 mg/l (exceeding the range of the working sample concentrations) was analysed.

Linearity was confirmed ($R^2 = 1$) in the range 0 to 51 mg/l.

The results are presented graphically on page 53.

3.2 Recoveries

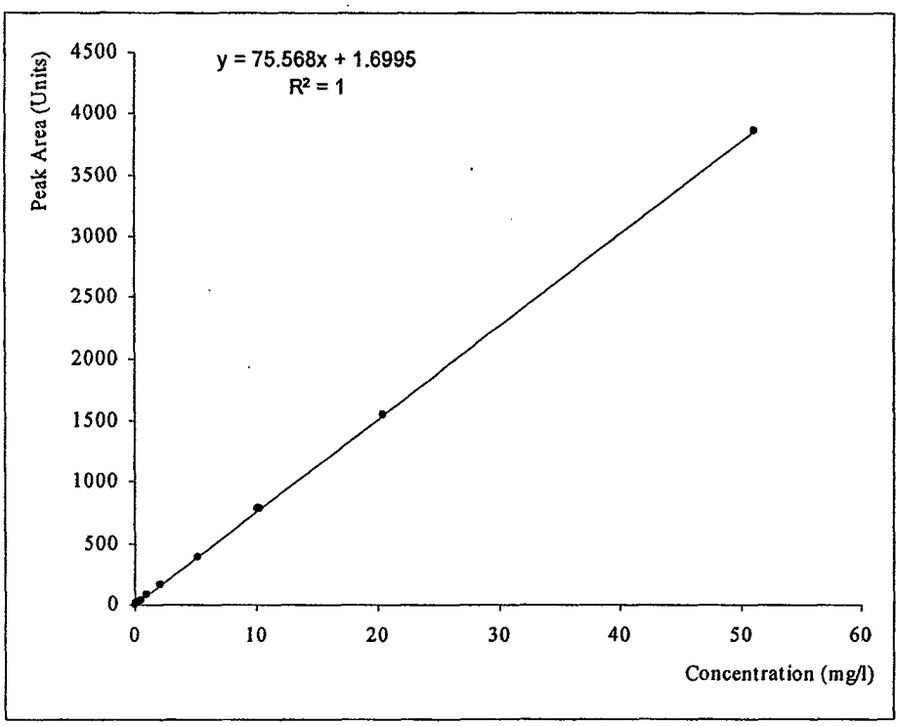
A range of preliminary test samples, accurately fortified at known concentrations of test material, was prepared and analysed.

LOQ = limit of quantitation

ALGAL GROWTH INHIBITION TEST

Appendix 3 (continued) Verification of Test Concentrations

Linearity of Detector Response



Appendix 3 (continued) Verification of Test Concentrations

The recovery samples were prepared by addition of a standard solution of test material to a sample of test medium. A standard solution was accurately prepared by dissolving the test material in methanol. An accurate volume of the standard solution was added to a known volume of test medium to achieve the required concentration of test material.

A further portion of a test sample was analysed following the addition of algal cells to assess the effects of algae on the recovery of test material from test medium.

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
0.0158	0.0133	84	80
0.0158	0.0121	77	
0.0158 plus algae	0.0161	102	-
0.158	0.146	93	91
0.158	0.141	89	
1.58	1.37	86	85
1.58	1.31	83	
1.58 plus algae	1.35	85	-

The method has been considered to be sufficiently accurate for the purposes of this test. The test sample results have not been corrected for recovery.

The presence of algal cells was considered to have no significant effect on the recovery of the test material from the medium.

3.3 Limit of Quantitation

The limit of quantitation has been assessed down to 0.00022 mg/l.

4. STABILITY

A range of preliminary test samples was prepared, analysed initially and then after storage in sealed glass vessels at ambient temperature in light and dark conditions for approximately 72 hours (equivalent to the test exposure period). In addition test samples were tested for stability

Appendix 3 (continued) Verification of Test Concentrations

without prior mixing (sonication) of the test sample bottles to assess for losses due to adsorption and/or insolubility.

Nominal concentration (mg/l)	0.015	0.15	1.5
Concentration found initially (mg/l)	0.0127	0.144	1.34
Concentration found after storage in light conditions (mg/l)	0.00575	0.101	1.19
Expressed as a percent of the initial concentration	45	70	89
Concentration found after storage in dark conditions (mg/l)	0.00406	0.105	1.31
Expressed as a percent of the initial concentration	32	73	98
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	0.00421	NA	1.35
Expressed as a percent of the initial concentration	33	-	101

The test samples have been shown to be stable in the test medium at the highest level only. At the other two levels the test samples showed a significant decrease in concentration. The reason for this was unknown. Therefore as a precaution, all test samples were prepared under non-actinic light and amber vials were used.

The unsonicated stability vessel showed no evidence of insolubility or adherence to glass.

NA = Not applicable

ALGAL GROWTH INHIBITION TEST

Appendix 3 (continued) Verification of Test Concentrations

5. RESULTS

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
0 hours	Control	<LOQ	-
	0.015	0.0126	84
	0.048	0.0578	121
	0.15	0.142	95
	0.48	0.555	116
	1.5	1.81	120
72 hours	Control	<LOQ	-
	0.015	0.000269	2
	0.048	0.000353	<1
	0.15	0.000723	<1
	0.48	0.332	69
	1.5	1.06	71

6. DISCUSSION

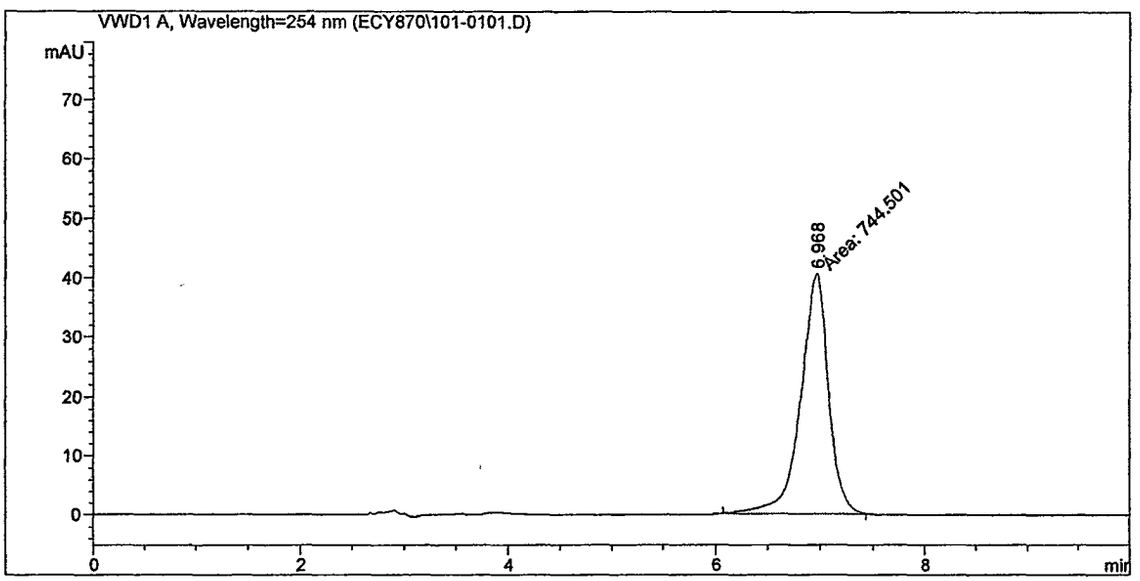
The detection system was found to have acceptable linearity. The analytical procedure had acceptable recoveries of test material in test medium. A method of analysis was validated and proven to be suitable for use.

LOQ = Limit of quantitation

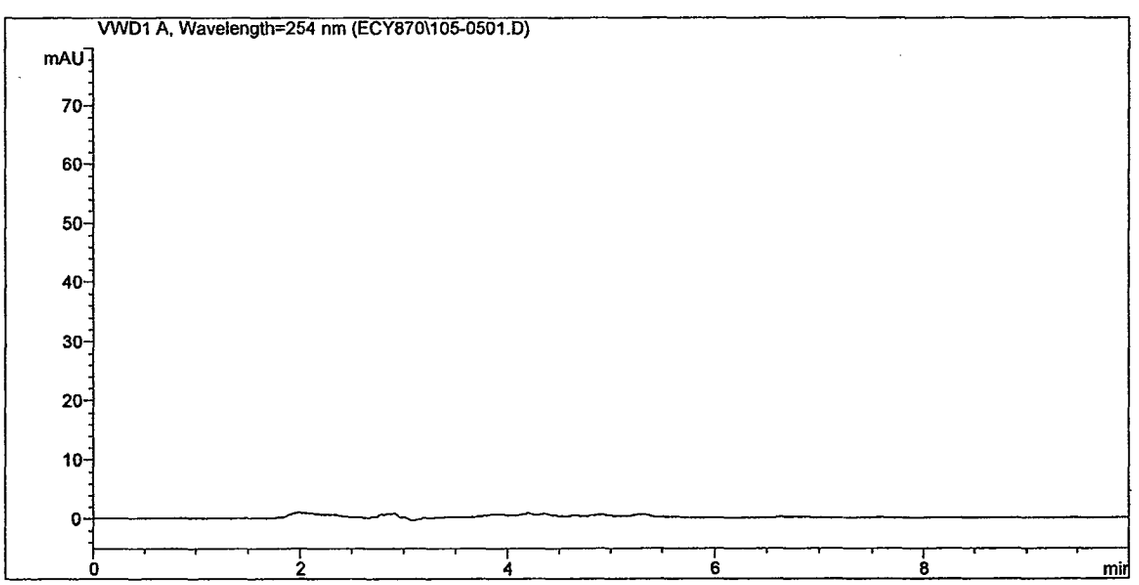
ALGAL GROWTH INHIBITION TEST

Appendix 3 (continued) Verification of Test Concentrations

7. TYPICAL CHROMATOGRAPHY



Standard 10 mg/l 0 Hours



Control Sample 0 Hours

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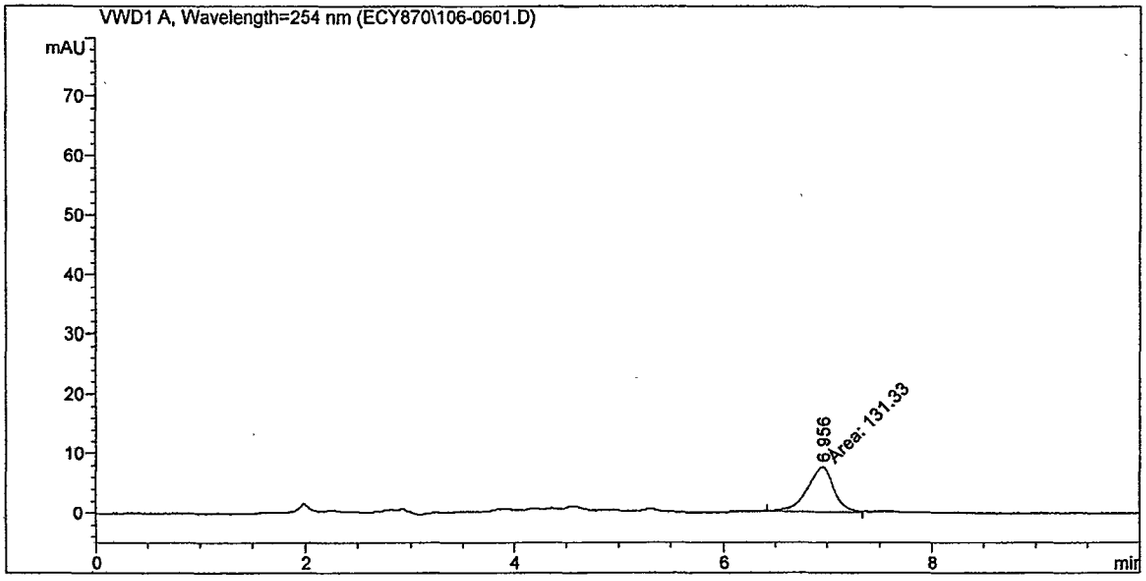
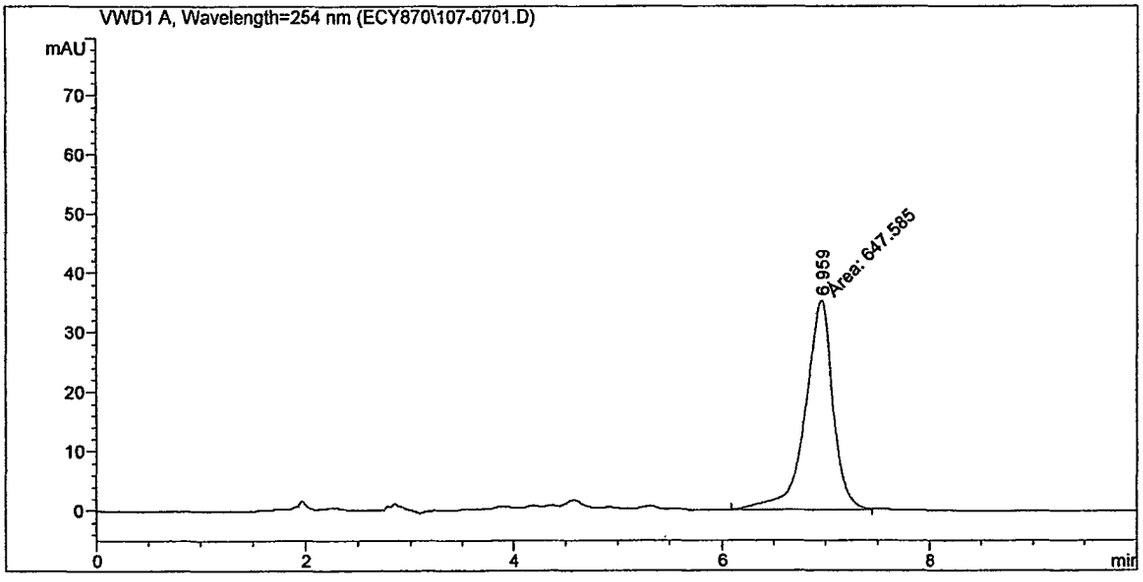
Appendix 3 (continued) Verification of Test Concentrations



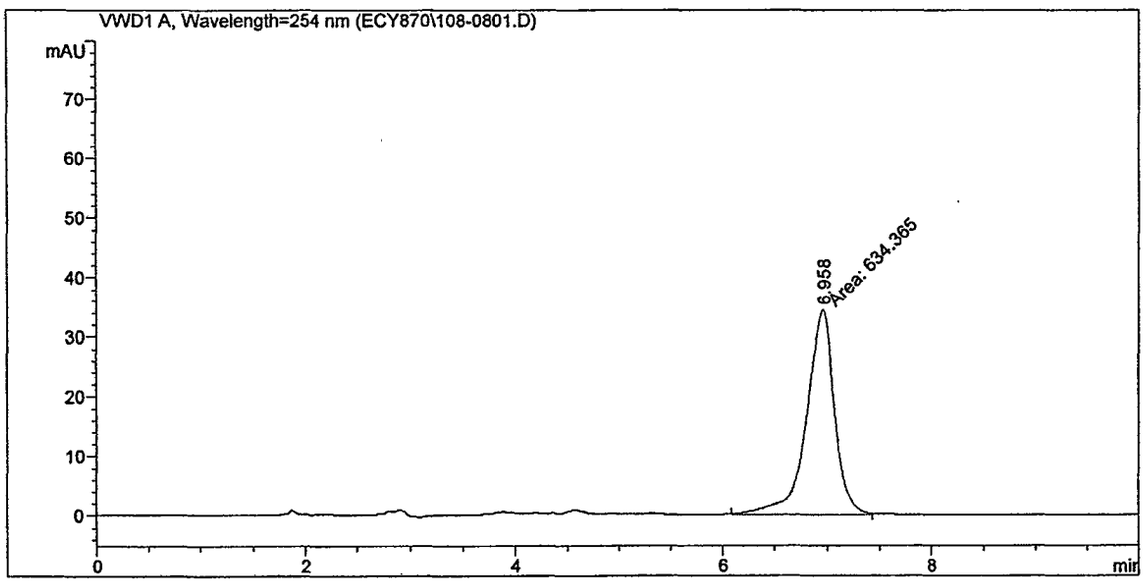
Test Sample 0.015 mg/l 0 Hours



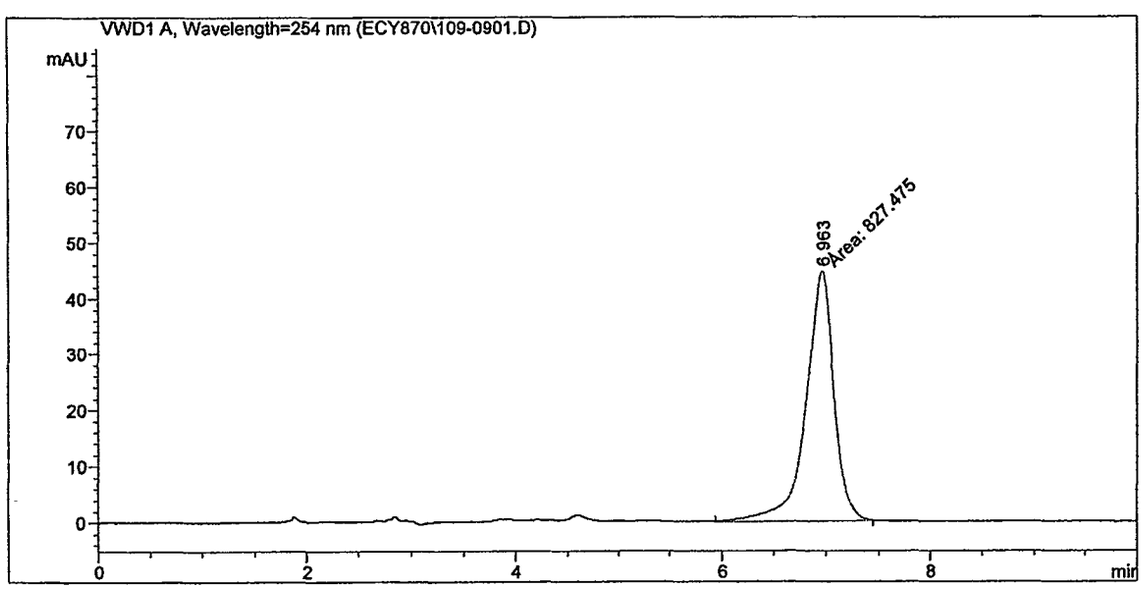
Test Sample 0.048 mg/l 0 Hours

ALGAL GROWTH INHIBITION TEST

Appendix 3 (continued) Verification of Test Concentrations



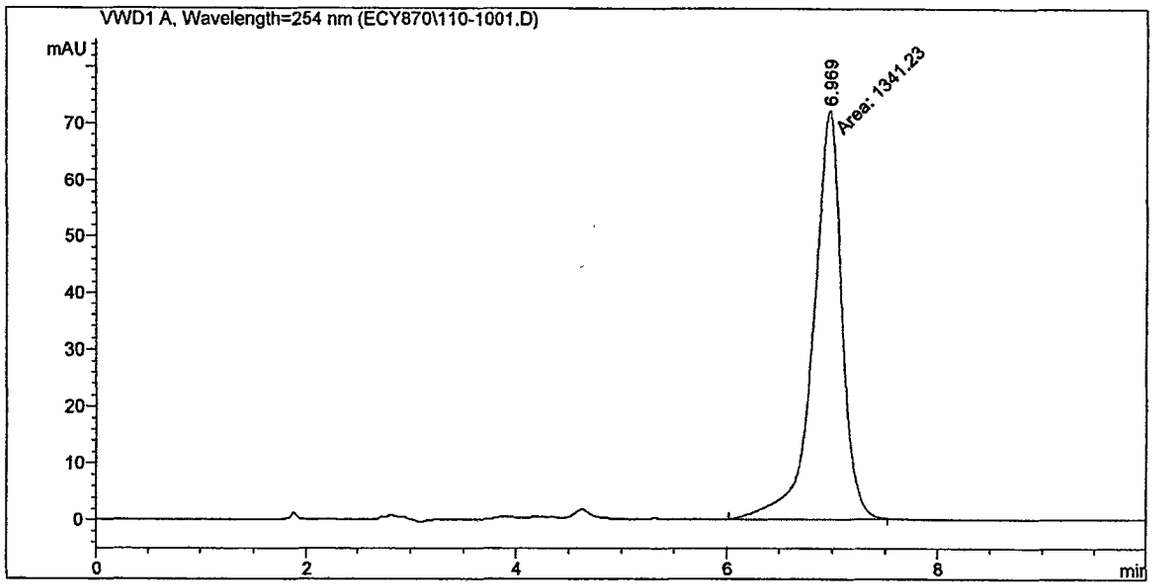
Test Sample 0.15 mg/l 0 Hours



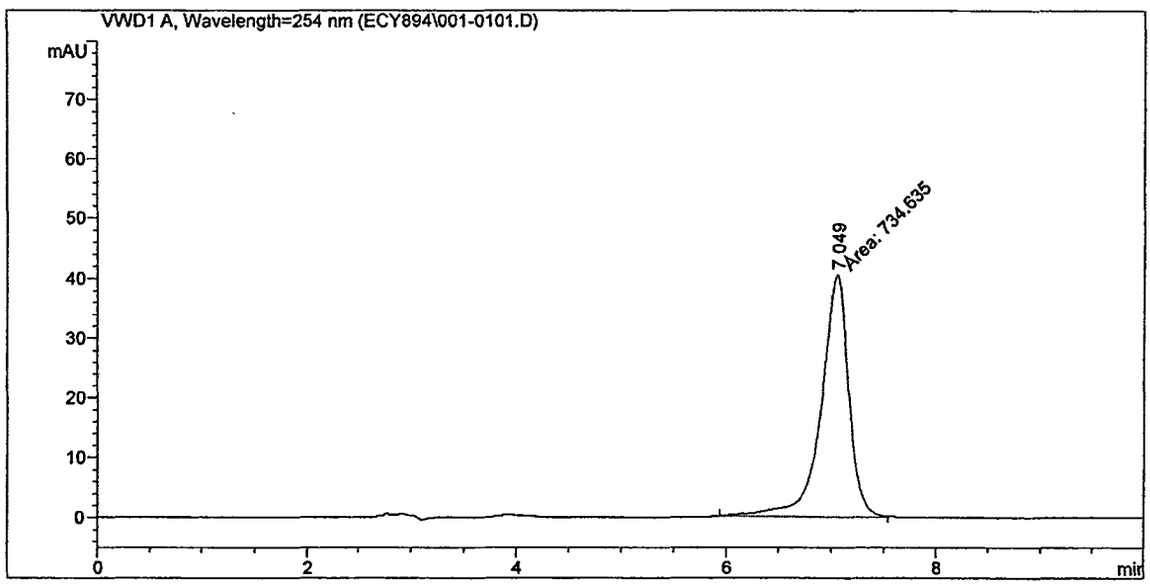
Test Sample 0.48 mg/l 0 Hours

ALGAL GROWTH INHIBITION TEST

Appendix 3 (continued) Verification of Test Concentrations



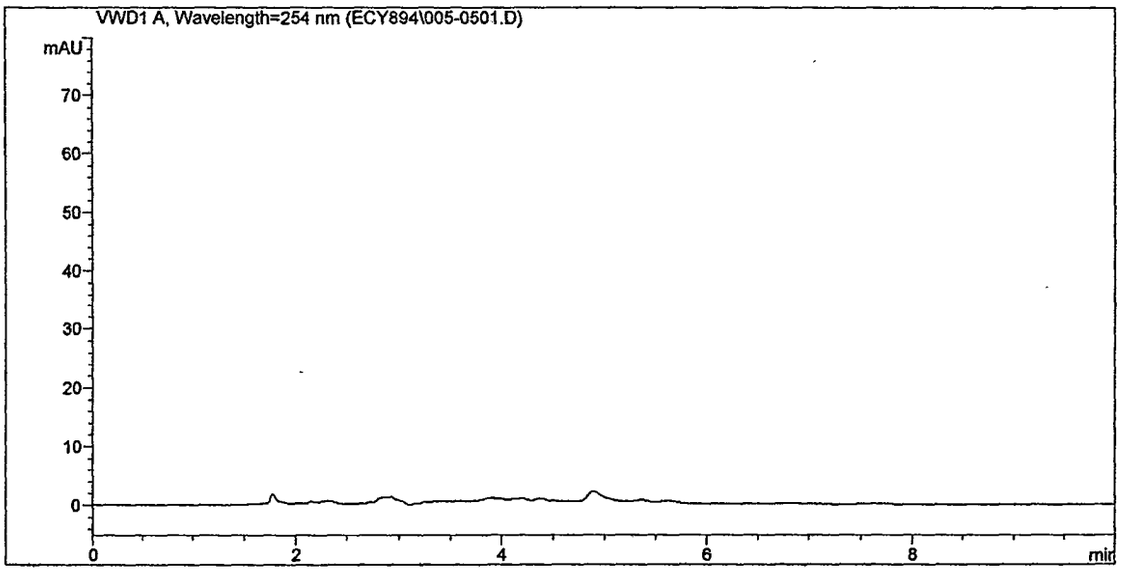
Test Sample 1.5 mg/l 0 Hours



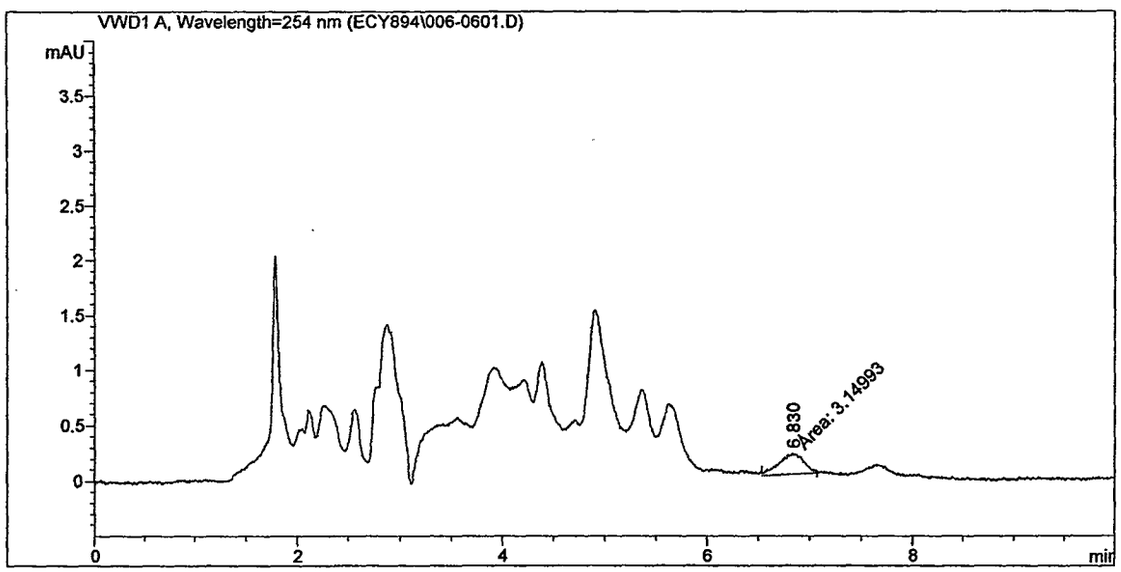
Standard 10 mg/l 72 Hours

ALGAL GROWTH INHIBITION TEST

Appendix 3 (continued) Verification of Test Concentrations



Control Sample 72 Hours



Test Sample 0.015 mg/l 72 Hours

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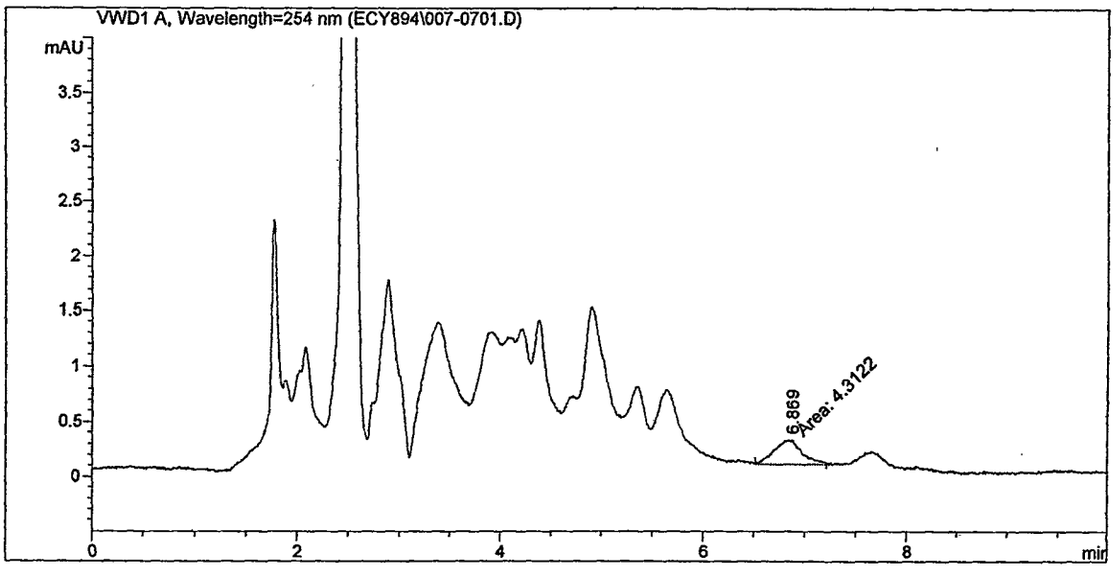
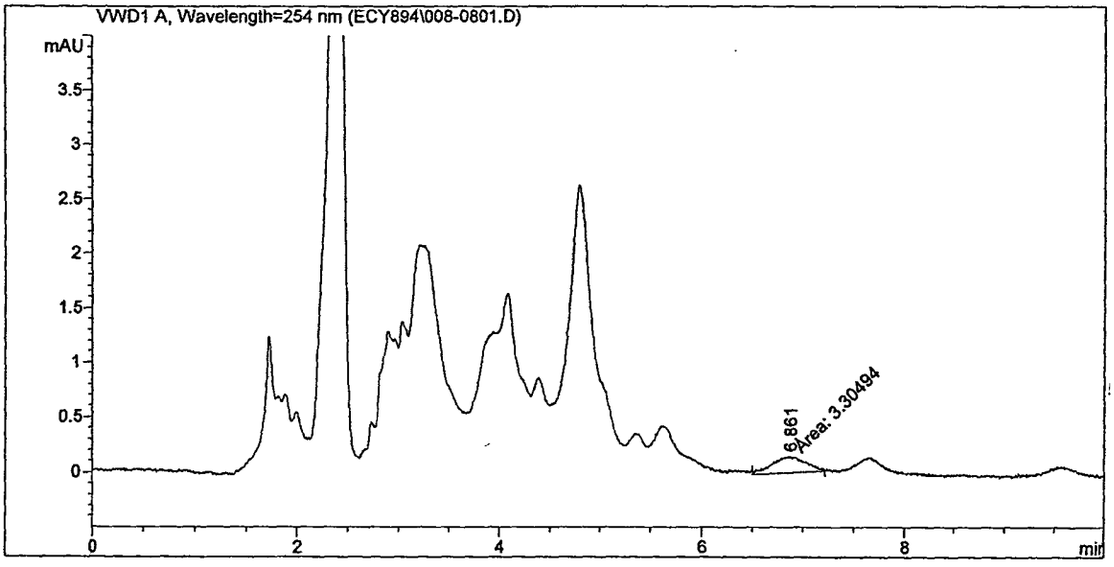
Appendix 3 (continued) Verification of Test Concentrations



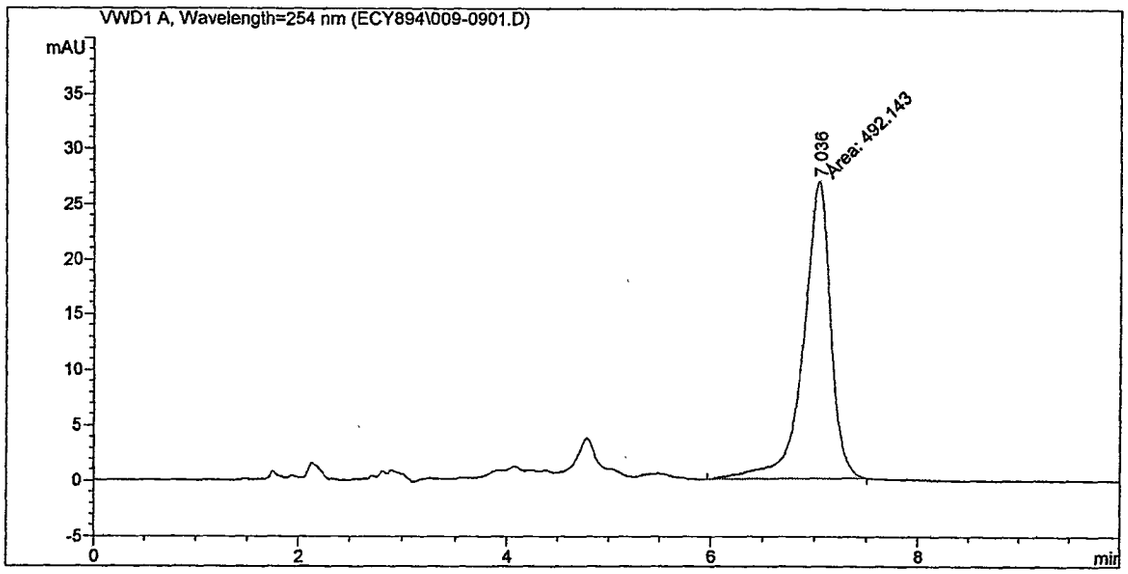
Test Sample 0.048 mg/l 72 Hours



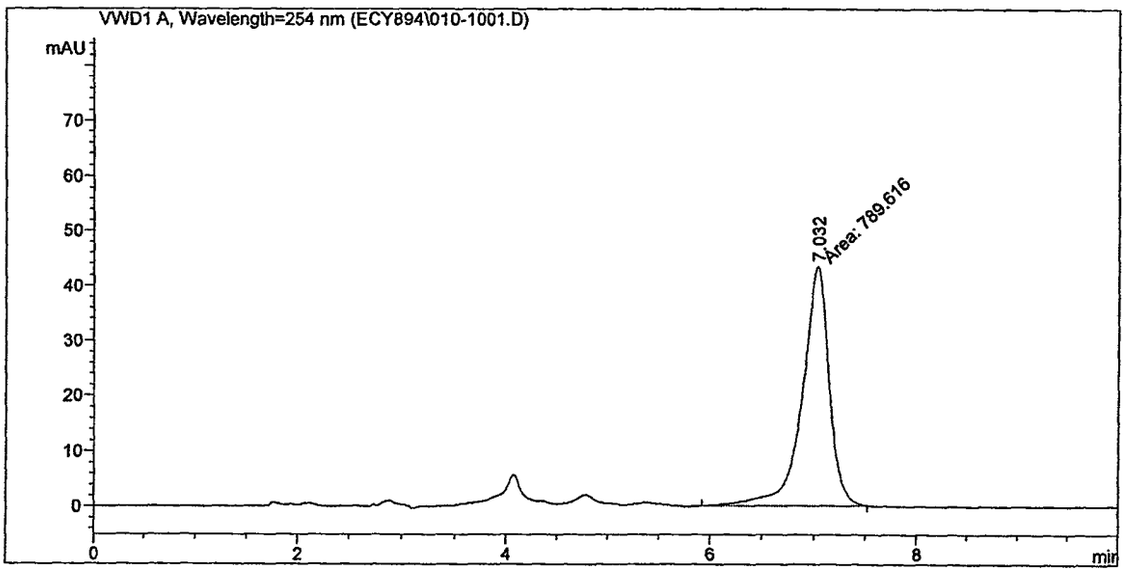
Test Sample 0.15 mg/l 72 Hours

ALGAL GROWTH INHIBITION TEST

Appendix 3 (continued) Verification of Test Concentrations



Test Sample 0.48 mg/l 72 Hours



Test Sample 1.5 mg/l 72Hours

Appendix 4 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

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DH Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY	TEST TYPE
SafePharm Laboratories Ltd. **Shardlow Business Park** **Shardlow** **Derbyshire** **DE72 2GD**	**Analytical Chemistry** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem testing** **Toxicology**

DATE OF INSPECTION

21st August 2007

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

15/10/07

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA